

Production of fish protein hydrolysates by a marine proteolytic strain

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Abstract

The preparation of fish protein hydrolysates by a fermentative process is an alternative to classical preparation using added proteolytic enzymes, taking advantage of the wide spectrum of proteolytic enzymes produced by microorganisms and increasing the complexity of the peptide mixture obtained. Thus, the objective of this work was to prepare fermentative protein hydrolysates (FPH) from a commercial cod protein hydrolysate (CPH) using an indigenous marine proteolytic strain. The bacterial growth was performed in liquid culture media, containing three concentrations of CPH (1, 2 and 4%), yeast extract (0.5%) and sucrose (0.5%), with continuous shaking, at 30 °C for 24, 50 and 72 hours. The parameters evaluated were the culture growth, enzymatic activity, protein content and recovery, peptide profile, degree of hydrolysis and antioxidant activity, measured by DPPH and ABTS radical scavenging activity, reducing power and metal chelating activity. Bacterial growth and enzymatic activity reached their maximums after 24 hours of fermentation, and all FPH showed the disappearance of peptides in the range of 200 – 400 Da. In the set of samples, 4 % FPH recorded the highest values of protein content and recovery, but 1 % and 2 % FPH were the samples that presented improved antioxidant activity.

Key-words: Fermentative protein hydrolysate, proteolytic strain, fish by-products, antiradical activity, chelating activity, reducing power.

Resumo

A preparação de hidrolisados de proteicos de pescado através de um processo fermentativo é uma alternativa à preparação clássica utilizando enzimas proteolíticas adicionadas, aproveitando o amplo espectro de enzimas proteolíticas produzidas por microorganismos e o aumento da complexidade da mistura de péptidos obtidos. O objetivo deste estudo foi preparar hidrolisados proteicos fermentativos (FPH) a partir de um hidrolisado proteico de bacalhau comercial (CPH), utilizando uma estirpe proteolítica marinha. O crescimento bacteriano foi realizado em meios de cultura contendo três concentrações de CPH (1, 2 e 4 %), extrato de levedura (0,5%) e sacarose (0,5%), com agitação contínua a 30 ° C durante 24, 50 e 72 horas. Os parâmetros avaliados foram o crescimento bacteriano, atividade enzimática, recuperação e teor de proteína, perfil de péptidos, grau de hidrólise e atividade antioxidante, medida pela capacidade de captar os radicais DPPH e ABTS, poder redutor e atividade quelante. O crescimento bacteriano e atividade enzimática atingiram os valores máximos após 24 horas de fermentação, e todos FPH revelaram o desaparecimento de peptídeos na gama de 200-400 Da. No conjunto de amostras, 4 % FPH registou os maiores valores de recuperação e teor de proteína, enquanto 1 % e 2% FPH apresentaram melhor atividade antioxidante.

Palavras-chave: Hidrolisados proteicos fermentativos, estirpe proteolítica, subprodutos da pesca, atividade anti-radicalar, atividade quelante, poder redutor.

Resumo Alargado

Os processos de fermentação que ocorrem em vários tipos de produtos alimentares proteicos podem levar à formação de misturas muito complexas de péptidos. Tal fato resulta de uma ampla gama de proteases extracelulares produzidas pelos microrganismos. A produção de hidrolisados proteicos de pescado tem sido considerada uma boa alternativa para a valorização dos subprodutos da pesca. Estes hidrolisados proteicos são geralmente preparados por adição de proteases comerciais, embora o processo de fermentação não tenha sido utilizado na sua produção. Assim, o objetivo deste trabalho foi preparar hidrolisados proteicos fermentativos (FPH) a partir de um hidrolisado proteico de bacalhau comercial (CPH), utilizando uma estirpe bacteriana proteolítica de origem marinha, presuntivamente *Bacillus* sp..

O crescimento bacteriano foi realizado em meios de cultura líquidos, contendo três concentrações de CPH (1 %, 2 % e 4 %), extrato de levedura (0,5 %) e sacarose (0,5 %), com agitação contínua, a 30° C durante 24, 50 e 72 horas. Avaliou-se o crescimento bacteriano por medição da absorvância e contagens de unidades formadoras de colónias (CFU). Após cada período de fermentação, os meios de cultura foram centrifugados (10 000 rpm, 15 minutos) e os sobrenadantes foram filtrados e identificados como 1 % FPH, 2 % FPH e 4% FPH. Foram, ainda, retiradas alíquotas de FPH para a determinação da actividade enzimática e perfil de péptidos. Os FPH líquidos foram concentrados e liofilizados e determinadas as respectivas actividades antioxidante e quelante.

As contagens de colónias e máximos de actividade enzimática foram obtidos após 24 horas. Após 72 horas de fermentação os teores de proteína, em percentagem de peso seco, foram cerca de 40 %, 50 % e 62 %, para 1 %, 2 % e 4 % FPH, respetivamente. Os rendimentos obtidos com base no teor em proteína foram de 29 %, 28 % e 58 % para 1 %, 2 % e 4 % FPH, respectivamente. O aumento mais elevado (6 %) do grau de hidrólise foi observado em 2 % FPH após 72 horas de fermentação. Registaram diferenças importantes perfil de péptidos relativamente inicial, ocorrendo o desaparecimento de peptídeos na gama de 200- 400 Da e formação de dipéptidos e aminoácidos livres. A actividade de eliminação do radical livre DPPH dos FPH aumentou com o período de fermentação, que se traduziu pela diminuição do valor de EC_{50} . A actividade anti-radicalar dos diferentes FPH em relação ao ABTS não mostrou uma tendência clara em função do período de fermentação ou da concentração de FPH. Quanto ao poder redutor, o valor mais elevado foi atingido após 72 horas de fermentação, sendo mais elevado no 1 % FPH. No entanto, o poder redutor diminuiu com o aumento da concentração de HPF. No que diz respeito à actividade quelante, os FPH obtidos após qualquer período de fermentação mostraram menor actividade quelante, tanto do Cu^{2+} , como do Fe^{2+} , do que os péptidos iniciais. Apesar destes decréscimos na

atividade quelante dos FPH, estes apresentaram maior capacidade ligante em relação ao Cu^{2+} comparativamente ao Fe^{2+} .

LIST OF CONTENTS

Chapter I - Introduction	1
1. Objectives.....	1
2. Literature Review	1
2.1. Fish industry and by-products valorization	1
2.2. Fish protein hydrolysates	3
2.2.1. Production of fish protein hydrolysates.....	3
2.2.2. Chemical composition of fish protein hydrolysates	9
2.2.3. Properties of fish protein hydrolysates	9
2.2.4. Applications of fish protein hydrolysates	13
 Chapter II - Materials and Methods.....	16
1. Materials.....	16
2. Methods	16
2.1. Hydrolysates production.....	16
2.1.1. Preparation of culture media and microbiological assay.....	16
2.1.2. Optical Density (OD) measurements and separation of the bacteria from the medium	17
2.1.3. Supernatant storage, concentration and freeze-drying	17
2.2. Hydrolysates characterization.....	17
2.2.1. Determination of enzymatic activity	17
2.2.2. Gel filtration and peptide profile	18
2.2.3. Protein content determination	19
2.2.4. Yields of protein recovery	19
2.2.5. Degree of hydrolysis (DH).....	19
2.3. Antioxidant properties of FPH	20
2.3.1. Antiradical activity.....	20

2.3.2. Reducing Power	22
2.3.3. Metal ion chelating activity	22
2.4. Statistical analysis	23
Chapter III - Results and Discussion	24
1. Microbial Growth and Protease Production	24
2. Evolution of Protein Content During Bacterial Growth and Degree of Hydrolysis	26
3. Molecular Weight Distribution	28
4. Antioxidant Activity of the FPH	31
4.1. DPPH radical scavenging activity	31
4.2. ABTS radical scavenging activity	35
4.3. Reducing Power	39
4.4. Copper chelating activity	43
4.5. Iron chelating activity	46
Chapter IV – Conclusion	51
1. Concluding Remarks.....	51
2. Future work	53
References.....	54
Appendixes.....	65
Appendix I. Standard curves	65
Appendix II. Molecular weights' index	67
Appendix III. FPLC chromatograms.....	70

LIST OF TABLES

Table 1. Brand names, sources/producing methods and health promoting applications of some commercially available nutraceuticals/health foods/functional foods produced from fish protein hydrolysates.	14
Table 2. Evolution of protein content (% DW) of FPH samples, as well as commercial CPH and YE used in their formulations. Means with the same letters indicates values not significantly different ($p > 0.05$), with A, B, C comparing the same fermentation time and a, b, c, d comparing the same FPH sample.	26
Table 3. DH (%) values of FPH samples. Means with the same letters indicates values not significantly different ($p > 0.05$), with A, B, C comparing the same fermentation period and a, b, c, d comparing the same FPH sample.	28
Table 4. Summary of initial and final values of antioxidant activity obtained in all FPH.	52
Table 5. List of MW of the peptides in 1 % FPH samples.....	67
Table 6. List of MW of the peptides in 2 % FPH samples.....	68
Table 7. List of MW of the peptides in 2 % FPH samples.....	69

LIST OF FIGURES

Figure 1. Diagram of fish proteins hydrolysates production by enzymatic hydrolysis. Adapted from Batista, 2011.	5
Figure 2. The microbial growth curve, with Log N (logarithmic number of microorganisms) as a function of time. Adapted from Adam & Moss, 2008.	7
Figure 3. Growth of <i>Bacillus</i> sp. strain in three culture media.	24
Figure 4. Effect of CPH concentration in the protease production. The same letter indicates values not significantly different ($p > 0.05$), with A, B, C comparing the same fermentation time and a, b, c comparing the same type of culture medium.	25
Figure 5. . Evolution of protein recovery in all FPH samples. The same letter indicates values not significantly different ($p > 0.05$), with A, B, C comparing the same fermentation time and a, b, c, d comparing the same type of culture medium	27
Figure 6. Gel filtration chromatogram showing the molecular weight distribution of hydrolysates at 72 hours of hydrolysis. Numbers represent the peaks' MW classes: 1 corresponds to ribonuclease A (13 700 Da), 2 corresponds to aprotinin (6 500Da), 3 corresponds to angiotensin I (1 296 Da), 4 corresponds to triglycine (189 Da) and 5 corresponds to glycine (75 Da). ① and ② represent the peaks corresponding to MW higher than 400 Da and MW between 200 and 75 Da, respectively.	29
Figure 7. Fractions of the FPH samples according to their molecular weight range for different times of fermentation, 0 hours (blue), 24 hours (green), 50 hours (red) and 72 hours (purple). SD values are not represented, since gel filtration was only performed once for each FPH.	30
Figure 8. DPPH radical scavenging activity of 1 %, 2 % and 4 % FPH at the initial time (0 hours), CPH and YE.	31
Figure 9. DPPH inhibition of 1 % FPH samples at different times of fermentation.	32
Figure 10. DPPH inhibition of 2 % FPH samples at different times of fermentation.	33

Figure 11. DPPH inhibition of 4 % FPH samples at different times of fermentation.	33
Figure 12. Time course of EC ₅₀ values of FPH samples. SD values were obtained by four combinations of absorbance values recorded during the DPPH test. The same letter indicates values not significantly different ($p > 0.05$), with A, B, C comparing the same fermentation time and a, b, c comparing the same FPH sample.	34
Figure 13. ABTS radical scavenging activity of 1 %, 2 % and 4 % FPH at the initial time (0 hours), CPH and YE.	35
Figure 14. TEAC of 1 % FPH at different times of fermentation.	36
Figure 15. TEAC of 2 % FPH at different times of fermentation.	37
Figure 16. TEAC of 4 % FPH at different times of fermentation.	37
Figure 17. Evolution of ABTS radical scavenging activity for all samples at 5 mg FPH mL ⁻¹ . The same letter indicates values not significantly different ($p > 0.05$), with A, B, C comparing the same fermentation time and a, b, c, d comparing the same FPH sample.	38
Figure 18. Ascorbic acid equivalent reducing power of 1 %, 2 % and 4 % FPH, CPH and YE at the initial time (0 hours) as a function of protein concentration.	39
Figure 19. Ascorbic acid equivalent reducing power of 1 % FPH at different times of fermentation.	40
Figure 20. Ascorbic acid equivalent reducing power of 2 % FPH at different times of fermentation.	41
Figure 21. Ascorbic acid equivalent reducing power of 4 % FPH at different times of fermentation.	41
Figure 22. Evolution of reducing power for all samples at 10 mg FPH mL ⁻¹ . The same letter indicates values not significantly different ($p > 0.05$), with A, B, C comparing the same fermentation time and a, b, c, d comparing the same FPH sample.	42

Figure 23. Cu^{2+} chelating activity of 1 %, 2 % and 4 % FPH at the initial time (0 hours), CPH and YE as a function of protein concentration.	43
Figure 24. Cu^{2+} chelating activity of 1 % FPH samples at different fermentation periods	44
Figure 25. Cu^{2+} chelating activity of 2 % FPH samples at different fermentation periods.	44
Figure 26. Cu^{2+} chelating activity of 4 % FPH samples at different fermentation periods.	45
Figure 27. Evolution of Cu^{2+} chelating activity for all samples in 1 mg FPH mL^{-1} . The same letter indicates values not significantly different ($p > 0.05$), with A, B, C comparing the same fermentation time and a, b, c, d comparing the same FPH sample.....	45
Figure 28. Fe^{2+} chelating activity of 1 %, 2 % and 4 % FPH, CPH and YE at the initial time (0 hours) as a function of protein concentration.	47
Figure 29. Fe^{2+} chelating activity of 1 % FPH samples at different times of fermentation.....	47
Figure 30. Fe^{2+} chelating activity of 2 % FPH samples at different times of fermentation.....	48
Figure 31. Fe^{2+} chelating activity of 2 % FPH samples at different times of fermentation.....	49
Figure 32. Evolution of Fe^{2+} chelating activity for all samples, when concentration is 10 mg FPH mL^{-1} . The same letter indicates values not significantly different ($p > 0.05$), with A, B, C comparing the same fermentation time and a, b, c, d comparing the same FPH sample.....	49
Figure 33. Calibration curve for Superdex™ peptide 10/300 GL, and its respective equation ($y = -0.2527x + 1.1909$, $R^2=0.989$, where y represents the k_{av} coefficient and x represents the logarithmic value of MW).	65
Figure 34. Trolox calibration curve and its respective equation ($y = 0.0361x - 4.0742$, $R^2=0.999$, where y represents the percentage of inhibition, or disappearance of ABTS, and x represents the concentration of Trolox).	66
Figure 35. Ascorbic acid standard curve and its respective equation ($y = 10.559x + 0.0037$, $R^2= 0.9992$, where y represents the absorbance and x the concentration of ascorbic acid). 66	

Figure 36. Gel filtration chromatograms showing the molecular weight distribution of hydrolysates at 0, 24 and 50 hours of fermentation.	70
Figure 37. Gel filtration chromatograms showing the evolution of molecular weight distribution of 1 %, 2 % and 4 % FPH.	71
Figure 38. Gel filtration chromatogram showing the molecular weight distribution of YE.	71
Figure 39. Gel filtration chromatogram showing the molecular weight distribution of CPH...	71

LIST OF ABBREVIATIONS

ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

CFU – Colony-forming Units

CPH – Cod Protein Hydrolysate

DH – Degree of Hydrolysis

DPPH – 2,2 – Diphenyl – 1 – picrylhydrazyl

DW – Dry Weight

EC₅₀ – Half Minimal Effective Concentration

Ferrozine – 3 – (2 – Pyridyl) – 5,6 – bis (4 – phenyl – sulphonic acid) – 1,2,4 - triazine

FITC – Fluorescein isothiocyanate

FPH – Fermentative Protein Hydrolysate

FPLC – Fast Protein Liquid Chromatography

MW – Molecular Weight

OD – Optical Density

OPA – *o* – Phthaldialdehyde

PCA – Plate Count Agar

PV – Pyrocatechol Violet

ROS – Reactive Oxygen Species

TCA – Trichloroacetic Acid

TEAC – Trolox Equivalent Antioxidant Capacity

TFA – Trifluoroacetic Acid

Tris – Tris - (hydroxymethyl) - aminomethane

TSA – Tryptic Soy Agar

YE – Yeast Extract

CHAPTER I - INTRODUCTION**1. OBJECTIVES**

The purpose of the present study was to produce fermentative protein hydrolysates from a commercial cod protein hydrolysate, using a marine proteolytic strain, presumptively a *Bacillus* sp.. The work consisted in perform the bacterial growth under controlled conditions of shaking and temperature, in liquid culture medium with different concentrations of cod protein hydrolysate (1 %, 2 % and 4 %), yeast extract (0.5 %) and sucrose (0.5 %), at three different fermentation periods: 24, 50 and 72 hours.

It was also an objective the evaluation of the new hydrolysates about their protein content, protein yield, degree of hydrolysis, molecular weight distribution and antioxidant capacity (using five different methodologies), in order to verify in which extent the fermentation period and concentration of cod protein hydrolysate influenced the aforementioned properties and characteristics.

2. LITERATURE REVIEW**2.1. Fish industry and by-products valorization**

Fish is a very important commodity and an essential source of protein in many countries worldwide. Halweil & Nierenberg (2008) estimated that worldwide one billion people depend on producing, processing and trading fish for their subsistence. Over the last decades, fish provision has transformed in the context of globalization, as fishermen are producing more fish for consumers living at large distances and consumers increasingly consume fish and fish products (Oosterveer, 2008). With the increasing of world population and a world catch of fish on the verge of exceeding the estimated sustainable limits of the suggested 100 million tons/year, there is an increasing need to utilize sea resources with

more efficiency and foresight, as well as it has led to an increase in aquaculture production (Kristinsson & Rasco, 2000; Cardoso & Nunes, 2013).

According to FAO (2012), capture fisheries and aquaculture supplied the world with about 148 million tons of fish in 2010, of which about 86 % was utilized as food, and preliminary data for 2011 indicate increased production of 154 million tons, of which 85 % was destined as food. In 2009, fish accounted for 16.6 % of the world population's intake of animal protein and 6.5 % of all protein consumed. Globally, fish provides about 3.0 billion people with almost 20 % of their intake of animal protein, and 4.3 billion people with about 15 % of such protein.

The fishing industry rejects approximately 75 % of total weight, although this amount of waste still has many components that could be reutilized. These large quantities of waste can create serious pollution and disposal problems, whereas some of the discarded parts are usually composted or thrown again in the ocean. The discards' portions that are returned to the sea are generally died or dried when returned to the ocean, which can lead to alterations on marine trophic chains (Bozzano & Sardà, 2002). Besides, fish industry is made up of many small business scattered in space, as well as supermarkets and hypermarkets which generate large amounts of waste, creating logistical problems and difficulties in setting wasted recovery units (Penven *et al.*, 2013).

According to their origin, fishing discards can be classified as wastes of fish processing, like guts, skin, scales and bones, and residual flesh and undervalued species (Benjakul & Morrissey, 1997; Hsu, 2010). Fish and fishery products represent a valuable source of protein and essential micronutrients for balanced nutrition and good health, and their wasted by-products are a good source of bioactive compounds. However, these wasted by-products and their potential application has been great subject of attention lately. The recovery of these by-products and using them as functional ingredients in food matrix is an exciting and promising alternative. Examples of these applications are the preparation of surimi, protein concentrates, fish-meal, fish oil and protein hydrolysates (Gildberg, 2001; Sucasas, 2011).

In order to utilize these fish processing by-product wastes, bioactive compounds can be extracted and purified with various technologies, by helping the improvement of human health and solving the pollution and disposal problems as well (Šližytė *et al.*, 2009; Chalamaiah *et al.*, 2012). Proper utilization of protein rich fish processing wastes and underutilized fish species could be achieved by converting these materials into fish protein hydrolysates.

2.2. Fish protein hydrolysates

Proteins in human diet are an acknowledged source of physiologically active components, which have a positive impact on the body's function after gastrointestinal digestion. The production of bioactive peptides can be achieved by solvent extraction, enzymatic hydrolysis and microbial fermentation of food proteins (Kim & Wijesekara, 2013).

Protein hydrolysates are defined as proteins that are chemically or enzymatically broken down into free amino acids and/or peptides, which can present a large range of molecular weight depending on the greater or lesser degree of hydrolysis (Kristinsson & Rasco, 2000). Nowadays, fish protein hydrolysates with good nutritional composition, amino acid profile, and antioxidant activities have gained great attention of food scientists. Protein hydrolysates are used as readily available sources of protein for humans and animals due to their good functional properties (Neklyudov *et al.*, 2000).

Due to the increasing interest of consumers in finding antioxidants from natural sources, which may have less potential hazard than synthetic ones, research on fish protein hydrolysates exerting antioxidant activity has gained a great interest. Antioxidants are generally employed to prevent lipid oxidation in foods, which avoids the formation of toxic compounds and undesirable odors and flavors (Lin & Liang, 2002). Biological and physico-chemical properties of hydrolysates depend on the protein substrate used, enzyme specificity, hydrolysis conditions and degree of hydrolysis (Rafik *et al.*, 2010).

2.2.1. Production of fish protein hydrolysates

Chemical hydrolysis of fish proteins

Chemical hydrolysis of fish proteins is achieved by cleaving peptide bonds in a relatively inexpensive and simple way. However, there are limitations to use protein hydrolysates obtained by this method in food ingredients, since it is a difficult process to control and leads to products with variable chemical composition and functional properties. The hydrolysis performed with strong chemicals and solvents at extreme temperatures and pH conditions may generate products with reduced nutritional qualities, restricted usage as flavor enhancers and poor functionality (Kristinsson & Rasco, 2000).

Acid hydrolysis

Acid hydrolysis of fish protein achieves higher yields and involves reaction of fish proteins with hydrochloric or sulfuric acid, at high temperature (and often high pressure), leading to a complete hydrolysis of proteins. Then, the hydrolysate is neutralized with sodium hydroxide to pH 6.0 to 7.0, which can lead to a formation of a large amount of salt (Thakar *et al.*, 1991, Batista, 2011). The salt formation in the neutralization stage can make the product unpalatable and interfere with functionality in food systems; furthermore it leads to high ash content in the obtained hydrolysates. Another disadvantage of acid hydrolysis is the destruction of amino acids, particularly tryptophan, which is an essential amino acid. The acid hydrolysis is utilized to convert underutilized and secondary raw material from fisheries into fertilizer due to the low production cost and resulting extensive hydrolysis (Kristinsson & Rasco, 2000, Batista, 2011).

Alkaline hydrolysis

The use of alkaline reactants, usually sodium hydroxide, to hydrolyze proteins often results in poor functionality, and can adversely affect the nutritive value of the product. Alkaline hydrolysis has primarily used fish protein concentrate as initial substrate, being rapidly cleaved into large water-soluble polypeptides, followed by further degradation at a slower rate. However, alkali treatment can aid in modifying the properties of insoluble fish protein concentrate. Tannenbaum *et al.* (1970a, 1970b) developed a small-scale batch process that utilizes pH 12.5 and 95 °C for 20 minutes, resulting in a product with large peptides but with an improvement in functionality. Several detrimental reactions occur during the alkaline hydrolysis. When the hydrogen is abstracted from the alpha carbon of an amino acid, racemization of L-aminoacids occurs, producing D-aminoacids which are not absorbed by humans. Also, disulfide bonds are split with loss of amino acids like cysteine, serine and threonine. Some of these elimination and addition reactions may lead to the formation of toxic substances (e.g., lysinoalanine), that are highly reactive products having an inhibiting effect on proteolytic enzymes, reducing the rate of hydrolysis (Kristinsson & Rasco, 2000).

Enzymatic hydrolysis of fish proteins

Enzymatic hydrolysis of proteins can be performed using endogenous enzymes (proteases existing in fish) or exogenous enzymes, added to promote the hydrolytic process.

The enzyme specificity should be taken into consideration, once it strongly influences the molecular size and hydrophobicity of the hydrolysate (Kristinsson & Rasco, 2000, Batista, 2011). The hydrolysates production involves fish grinding and water addition. When endogenous enzymes are used, the process is prolonged and the protein hydrolysis requires longer control, not ensuring to obtain products with a uniform quality standards. As to enzymatic hydrolysis with exogenous enzymes, these can be selected according to the intended characteristics for the product (Batista, 2011). The hydrolysis occurs under moderate conditions, avoiding extreme conditions of pH and temperature, which can compromise the nutritive quality of the hydrolysate (Kristinsson & Rasco, 2000). In figure 1 is presented a preparation scheme of fish protein hydrolysate.

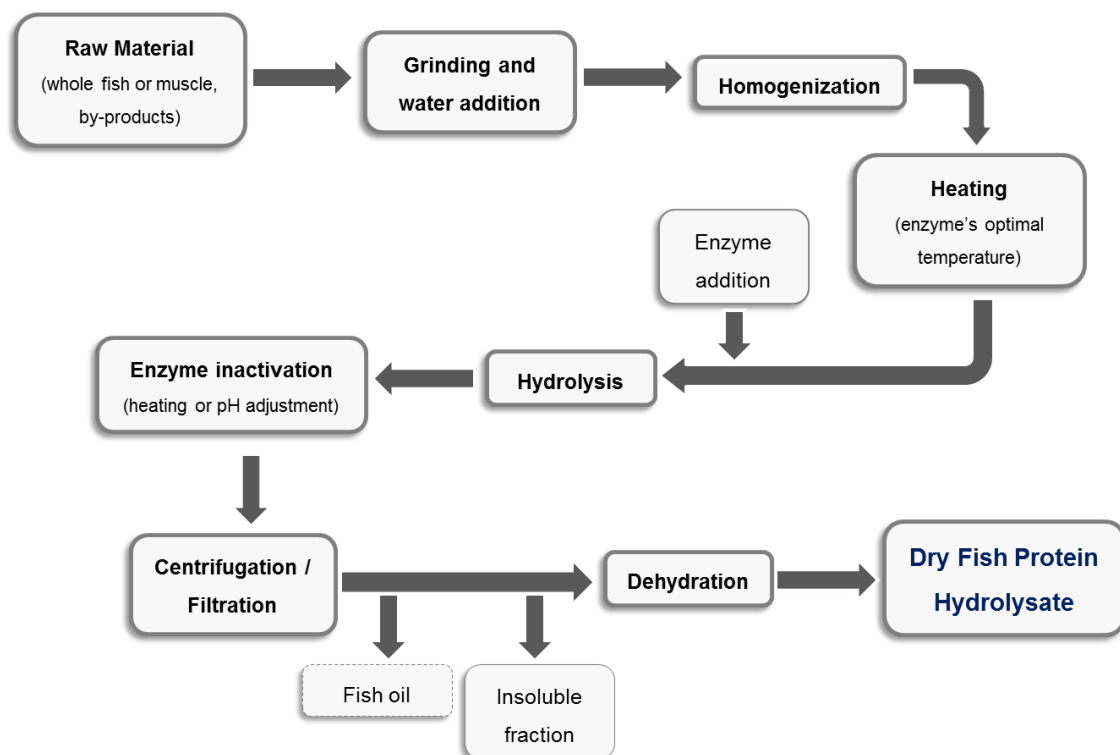


Figure 1. Diagram of fish proteins hydrolysates production by enzymatic hydrolysis. Adapted from Batista, 2011.

The fish protein hydrolysate consists on grinding the material, followed by the addition and homogenization. This homogenization is very important to allow an easy access of the enzymes to the proteins. Then, the mixture is heated to the optimal temperature for the occurrence of enzymatic reaction, followed by pH adjustment. The hydrolysis depends on the substrate concentration and the enzyme/substrate affinity, pH, temperature and time of reaction. Then, the enzymatic reaction is stopped by thermal (75 - 100 °C during 5 – 30

minutes) or chemical (by increase or decrease of pH) inactivation. Centrifugation of the mixture allows the recovery of the hydrolysates, resulting in two fractions: one aqueous phase, which contains the hydrolyzed material, and an insoluble phase. The dehydration of the aqueous supernatant is usually made resort a spray dryer (Batista, 2011; Kristinsson & Rasco, 2000).

Fermentative hydrolysis

The application of microbials, in addition to enzymatic hydrolysis, is an emerging process that provides a natural technology applicable to the production of bioactive peptides. The fermentative process for the production of protein hydrolysates requires natural or added microorganisms (starter cultures), that during their growth hydrolyze sugars and proteins available in their surrounding medium. The hydrolysis, carried out by extracellular microbial proteases, results in a wide range of peptides with different amino acid sequences with interesting bioactive properties. There is a wide variety of proteases that can be produced by fermentative hydrolysis, although their yields, i.e. the degree of hydrolysis (DH), depend upon the microorganism species which produces them, the nutritional composition and the growth conditions (Venugopal, 1994).

Microbial growth

Microbial growth is an autocatalytic process: no growth will occur without the presence of at least one viable cell and the rate of growth will increase with the amount of viable biomass present, and it follows an exponential rate (Adam & Moss, 2008). The rate of growth usually follows the trend showed in figure 2.

By analyzing the growth curve, three major phases can be distinguished. In the first, the lag-phase, the inoculum is adjusting to the new environment, synthesizing enzymes required for cleaving the nutrients in the medium, so there is no apparent growth. The exponential or logarithmic phase, which follows, is characterized by an increase in cell numbers. Accordingly, the slope of the growth curve in this phase will equal the microorganisms specific growth rate, which itself will depend on intrinsic and extrinsic factors. Lastly, the changes in the medium resulting from the exponential growth, bring this phase to an end as the nutrients have ran out, or inhibitory metabolites accumulate, leading the

culture to enter the stationary phase. The intrinsic parameters that influence microbial growth are pH, moisture content, oxidation-reduction potential and nutrient content, while the extrinsic factors are temperature, relative humidity, presence and concentration of gases (Adam & Moss, 2008).

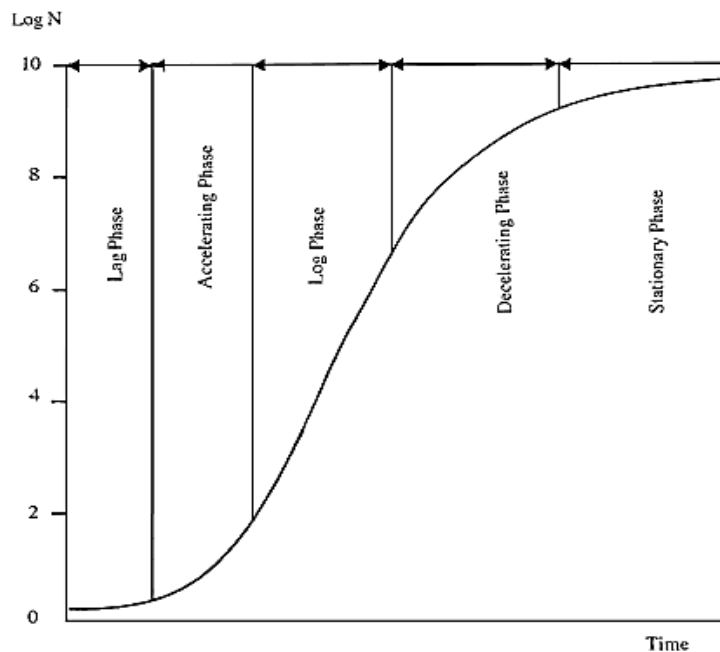


Figure 2. The microbial growth curve, with Log N (logarithmic number of microorganisms) as a function of time. Adapted from Adam & Moss, 2008.

Microbial proteases

For practical applications, proteases are produced from high-yielding microbial strains and under controlled conditions of fermentation. Depending upon the specific site of cleavage, these proteases can be divided into exoproteases, if they cleave the protein substrate at the N-terminal (aminopeptidases) or at the C-terminal (carboxyl peptidases) end; or endoproteases (or proteinases), if they cleave the peptides bonds within protein molecules, at specific residues to produce relatively large peptides, which give them a major industrial importance (Venugopal, 1994).

Apart from their sites of action on the protein, the enzymes are classified as acidic, neutral or alkaline, depending on the pH requirements for their optimal activities. Acid proteases are commonly produced by molds and yeasts, while bacteria secrete neutral or alkaline proteases. Based on their mechanism of action and sensitivities to various types of inhibitors, proteases can be classified in four major classes, which are designated by the

principal functional group in their active site: serine, thiol, metallo and aspartic proteases, which have serine, thiol, metal ion or aspartic acid, respectively, in their active sites (Venugopal, 1994, Kristinssom, & Rasco, 2000).

Serine proteinases are produced by bacteria, yeasts and molds and have nucleophilic serine residue in their active sites. Apart from this, these proteinases are also distinguished by having essential aspartate and histidine residues, and they include trypsin-like proteinases and alkaline proteinases. Trypsin-like proteinases have an optimum pH of 8 and may also possess esterase and amidase activities. These enzymes are commercially important, since they are used in detergent industry. Thiol proteinases reduce compounds like cysteine but are inhibited by oxidizing agents. Aspartic proteinases have several industrial applications and show specificity towards aromatic or bulky amino acid residues at both sides of the peptide bond. Their optimal pH is at 3 – 4 and are insensitive to inhibitors like difluorophosphate, parachloro mercurybenzoate and ethylenediamine tetraacetic acid. As for metalloproteinases, they involve a metal ion in their catalytic mechanisms, which can be either neutral or alkaline. Several bacteria and fungus produce this type of proteinases (Venugopal, 1994, Kristinssom, & Rasco, 2000, Gupta *et al.*, 2002).

The secretion of proteinases by the microorganisms occurs at minimal levels during their exponential growth, but increases with a decrease in nutrients and reaches a maximum towards the late exponential/early stationary phase (Venugopal, 1994).

Microbial proteases have great advantages in food application, which includes their low cost of production, less time required for enzyme isolation, possibilities for mass production and amenability of the organisms to genetic manipulations to improve the enzyme yields. The applications of these enzymes include production of oriental foods, bread-making, cheese ripening, meat tenderization and chill-proofing beer, as well as detergent industry and diagnostic purposes (Venugopal, 1994).

Uses of microbial proteases in the production of fish protein hydrolysates

Many authors have reported that microbial enzymes have an improved potential compared to plant enzymes, due to their ability to solubilize proteins. Nevertheless, it is essential that microorganisms used are safe, if the newly formed hydrolysates are intended for food applications (Venugopal, 1994). Kilara (1985) reported that neutral and alkaline proteases from bacteria were about 140 % more efficient than papain, and Hale (1969) showed that hydrolysis of raw fish with *Bacillus subtilis* protease at pH 8.5 gave high yield of soluble product, having an excellent balance of amino acids. In a more recent study, performed by Martínez-Alvarez *et al.* (2008), it was reported the production of highly active

CGRP-like molecules by enzymatic fish hydrolysis and subsequent treatment with *Saccaromices cerevisiae*. These results suggest that successful solubilization of fish proteins could be achieved by employing microbial proteases.

2.2.2. Chemical composition of fish protein hydrolysates

Chemical composition of fish protein hydrolysates is important to human health in a nutrition perspective, and it depends on the fish species and the enzymatic or chemical hydrolysis which originated them. Many researchers reported the protein content of these products ranged between 60 % to 90 % of its total composition (Dong *et al.*, 2005; Thiansilakul *et al.*, 2007; Souissi *et al.*, 2007; Ovissipour *et al.*, 2009; Khantaphant *et al.*, 2011). This high protein content of fish protein hydrolysates demonstrates its potential use as protein supplements for human nutrition.

The removal of lipids from the hydrolysates is very important, due to the development of unpleasant odors, rancidity and non-enzymatic browning caused by Maillard reactions (Batista, 2011). Overall, this stage of fish protein hydrolysates production leads to a fat content below 5 %, as it has been reported by Sathivel *et al.* (2005) and Souissi *et al.* (2007), among others.

Protein hydrolysates from various fish proteins contain moisture below 10 %, which is related to the type of sample and to the higher temperatures used during the process of evaporation and spray drying (Dong *et al.*, 2005; Kristinsson & Rasco, 2000; Foh *et al.*, 2011).

The relatively high ash content of fish protein hydrolysates is due to usage of added acid or base for adjustment of pH of reaction medium (Dong *et al.*, 2005; Choi *et al.*, 2009).

2.2.3. Properties of fish protein hydrolysates

Functional and physicochemical properties

The functional properties of fish proteins hydrolysates are important, especially if they're intended to use as ingredients in food industry, and are related to their molecular size, composition and amino acids sequence, electrostatic charges, water affinity, physicochemical properties of the parent protein used as substrate, protease specificity,

hydrolysis conditions and reaction time. However, the composition and processing parameters also interfere in the functional properties of hydrolysates (Pires & Batista, 2013).

Enzymatic hydrolysis of fish proteins generates a mixture of free amino acids, di-, tri- and oligopeptides, increasing the number of polar groups, which promotes the interaction with water, i.e. increased solubility, which also plays an important role in other functional properties, like foaming and emulsifying capacity (Chobert *et al.*, 1988; Mutilangi *et al.*, 1996, Kristinsson & Rasco, 2000).

The emulsifying capacity and stability of fish protein hydrolysates are related with the molecule hydrophilic and hydrophobic properties, giving them the capacity to constitute oil-water emulsions (Batista, 2011; Kristinsson & Rasco, 2000). Kristinsson & Rasco (2002) referred that the emulsifying properties generally decreased with the increase of the degree of hydrolysis, due to the formation of small peptides with less surface activity and less hydrophobicity. Previous studies (Klompong *et al.*, 2007) also found a connection between solubility and emulsifying capacity, as hydrolysates with high solubility can rapidly diffuse and adsorb at the interface.

Other functional property important for the application of protein hydrolysates in the food industry (especially meat and confectionery) is fat binding capacity, which depends on protein density, degree of hydrolysis, specificity of the hydrolyzing enzyme and hydrophobicity (Kinsella, 1976; Haque, 1993; Kristinsson & Rasco, 2000; Gbogouri *et al.*, 2004, Batista, 2011).

Foams are very similar to emulsions, i.e. they depend on the surface properties, and fish protein hydrolysates usually have good foaming capacity and make stable foams, due to their amphiphilic nature (Batista, 2011; Pires & Batista, 2013).

Proteins hydrolysis also modifies their ability to absorb and bind water molecules, giving a good ability to fish protein hydrolysates to bind and hold water. This is due to the increase of terminal carboxyl and amino groups, which produces a substantial effect on the amount of absorbed water and strength of the sorption bond, making them hygroscopic (Pires & Batista, 2013; Kristinsson & Rasco, 2000)

The bitterness of protein hydrolysates have been systematically reported by many authors, which constitutes a great disadvantage when their utilization in human food is intended. This unpleasant sensory characteristic has been attributed to the presence of hydrophobic amino acids, which are exposed by hydrolysis, and that may contact with taste buds (Batista, 2011). Nevertheless some studies have been made in an attempt to reduce the bitterness of fish protein hydrolysates, like those by Noguchi *et al.* (1975), which isolated acidic oligopeptides from fish protein hydrolysates that possessed a flavor-enhancing quality

like that of umami taste, and Jao & Ko (2002), who obtained tuna hydrolysates through ultrafiltration and reverse osmosis, also with aroma and umami taste.

Biological Properties

Bioactive peptides also exist in fish protein hydrolysates, although they are inactive when included in the parent protein. Nevertheless, their physiological activities can be exhibited when they are freed by hydrolysis during digestion or food processing (Batista, 2013).

A large number of peptides and amino acids prepared from food proteins have shown anticancer properties, although studies carried out with fish protein hydrolysate are very few. In a study performed by Picot *et al.* (2006), the antiproliferative activity of various commercial fish protein hydrolysates, of blue whiting, cod, plaice and salmon exhibited significant growth inhibitors against two human breast cancer cell lines. Bukowski (2003) also showed that peptides prepared from shark cartilage had antiangiogenic activity when they were tested *in vitro* and *in vivo*.

Antimicrobial peptides are small-molecular-weight peptides isolated and purified from living organisms, involved in natural defense mechanisms against pathogens. These peptides can be obtained from fish proteins by hydrolysis, like in the work performed by Liu *et al.* (2008), in which a cysteine-rich peptide isolated by digestion of oyster, with Alcalase and bromelain, presented antimicrobial activity against gram positive and negative bacteria, as well as fungus.

Hyperlipidemia (high levels of lipids and/or lipoproteins in blood) is one of the many causes of cardiovascular diseases, and many studies have been made in order to discover new peptides with hypocholesteremic activity. Wergedahl *et al.* (2004) prepared fish protein hydrolysates from salmon frames, which showed a cholesterol-lowering effect involving a decrease of plasma cholesterol in Zucker rats.

In fish protein hydrolysates obtained from cod, sardine and shrimp by-products has been reported the presence of peptides with low molecular weight that stimulate protein biosynthesis, control of intestinal motility and segregate digestive enzymes (Ravallec-Plé *et al.*, 2000).

Some fish protein hydrolysates are also provided with the ability to inhibit the action of the Angiotensin I Converting Enzyme (ACE), an enzyme present in many biological systems which exerts regulatory control over arterial blood pressure. The inhibition of ACE is one of the methods to reduce blood pressure, and some peptides have been reported to cause a similar effect on blood pressure such as synthetic drugs (Gálvez, *et al.*, 2013). It is thought

that ACE inhibition is related with the amino acid sequence, specially the tripeptide sequence at the C-terminal end. It is suggested that the presence of hydrophobic amino acids residues in this region favors the ACE inhibitory activity of the peptides, while hydrophilic peptides show weaker affinity for the ACE active sites (Ryan, 2011; Gálvez, *et al.*, 2013). In a study performed by Balti *et al.* (2010) with protein hydrolysates of cuttlefish, it was reported that the highest ACE inhibitory activity was displayed by the hydrolysate with the highest degree of hydrolysis.

Antioxidant Properties

One of the greatest concerns of food industry is the lipid oxidation, in which the resultant oxidized compounds are responsible for modifications in flavor (rancidification), color, texture, and even losses of nutritional value. Also, during cellular respiration of aerobic organisms (human included), reactive oxygen species (ROS), such as superoxide anion (O_2^-), singlet oxygen (O_2^*) and hydroxyl radicals ($\cdot HO$), along with free radicals and peroxides generated. Nevertheless, the prevention of their harmful effects, as well as of transition metals, is carried out by endogenous antioxidants, like enzymes, tocopherol, ascorbic acid and peptides. However, with advancing age, fatigue, excessive caloric intake, high fat diets and environmental pollutants, the balance between ROS, free radicals and antioxidants in human body can change. Beyond that, the attraction between unpaired electrons in valency of ROS and free radical and electrons from other substances, may lead to oxidative stress in the cells or tissues. For these reasons, is necessary to increase the amount of antioxidants in the human body, which led to a larger development of dietary antioxidant supplements and search for natural antioxidants (Zahara *et al.*, 2007; Batista, 2013).

Proteins can act as natural and multifunctional antioxidants by inhibiting several different oxidation pathways, including inactivation of ROS, scavenging free radicals, chelation of pro-active transition metal ions, like Fe^{2+} and Cu^{2+} , and nonradical reduction of hydroperoxydes to nonreactive hydroxydes (Jensen *et al.*, 2013). Peptides usually exhibit stronger antioxidant activity than free aminoacids due to their higher stability, which doesn't initiate or propagate further oxidative reactions (Elias *et al.*, 2008). Regarding free amino acids, their radical scavenging potential depends on its functional side chain, but they are limited by the tertiary structures of peptides or proteins, where they can be inaccessible to pro-oxidants. However, tertiary disruption can be achieved by food processing, fermentation or gastrointestinal digestion, freeing amino acids and allowing them to exhibit their antioxidant capacity (Jensen *et al.*, 2013).

Many authors referred that different amino acid residues may have their specific roles in peptides antioxidant activity, which can be by chelation of transition metals and/or scavenging free radicals. In a review presented by Chalamaiah *et al.* (2012), it is enhanced the importance of hydrophobic amino acids and one or more residues of histidine, proline, methionine, cysteine, tyrosine, tryptophan, valine, leucine and phenylalanine in the antioxidant activity of peptides. The presence of hydrophobic sequences in the peptides allows them to interact with lipid molecules and could scavenge by donating protons to lipid derived radicals. The aromatic amino acids, tyrosine and phenylalanine, as well as histidine, methionine and cysteine are indicated as direct radical scavengers. Tyrosine's antioxidant activity is due to the capability of phenolic groups to serve as hydrogen donors; the imidazole group in histidine has the proton-donation ability; methionine is prone to oxidation of the methionine sulfoxide; cysteine donates the sulfur hydrogen. The imidazole group in histidine has also been reported has metal chelator. Acidic and basic amino acids may also play an important role in Fe^{2+} and Cu^{2+} chelation (Carrasco-Castilla *et al.*, 2012).

In recent years, several studies have described the antioxidant activity of protein hydrolysates from fish sources, like yellowfin sole (Jun *et al.*, 2004), cobia skin (Yang *et al.*, 2008), tuna liver (Je *et al.*, 2009), sardinelle by-products (Bougatf *et al.* 2010) and backbone from Baltic cod (Zelechowska *et al.*, 2010).

2.2.4. Applications of fish protein hydrolysates

Food industry and nutritional value

Fish protein hydrolysates are good nutritional supplements as bioactive compounds and can be easily absorbed and utilized for various metabolic activities (Chalamaiah *et al.*, 2012). These peptides have potential in the food industry, especially protein supplements, bakery products, appetizers, cookies and soups. This application of fish protein hydrolysates resides in its capacity as flavor enhancers, due to their umami taste (Batista, 2011).

According to Chalamaiah *et al.*, (2012), in many countries, traditional and commercial preparations of fish protein hydrolysates are currently used as health nutraceutical foods. In table 1 is shown a short list of brand names, sources/producing methods and health promoting applications of commercially available nutraceuticals/health foods/functional foods in different countries, prepared from fish protein hydrolysates.

The usually balanced amino acid profile, good digestibility, quick intestinal absorption and bioactive peptides content, give fish protein hydrolysates a good nutritional value,

leading to their application in fish food. This inclusion of fish protein hydrolysates in fish food can improve the nitrogen retention and increase the feed conversion ratio for fish than food which contained only fish meal. In a study of Refstie *et al.* (2004) it was reported that the fish protein hydrolysate up to 15 % stimulated the consumption of food, increased the protein retention and digestibility as well as improved the growth of juvenile salmon (Batista, 2011).

Table 1. Brand names, sources/producing methods and health promoting applications of some commercially available nutraceuticals/health foods/functional foods produced from fish protein hydrolysates.

Product brand name	Sources / Producing methods	Nutraceutical applications	Country	Reference
Seacure®	Prepared by hydrolyzing deep ocean white fish	Dietary supplement helps to support the cells in the gastrointestinal tract and regulate bowel functions.	USA and Canada	Marchbank <i>et al.</i> (2008)
Amizate®	Produced from Atlantic salmon fish proteins by autolysis	Sports nutrition (supports the body's muscle anabolism and metabolic recovery).	North America	Nesse <i>et al.</i> (2011)
Stabilium® 200	Prepared from <i>Molva dypterygia</i> by autolysis	Supports the body's response to stress and provides nutritional support for memory and cognitive function	UK	Guerard <i>et al.</i> (2010)
PROTIZEN®	Produced by enzymatic hydrolysis of white fish proteins	It is "mood food" and dietary supplement to fight against stress and its symptoms (weight disorders, work pressure, sleep troubles, concentration difficulties and mood troubles).	UK	Guerard <i>et al.</i> (2010)

Adapted from Chilamaiah *et al.*, 2012

Microbial Culture Media

Several studies have been made in order to use fish protein hydrolysates in the place of commercial peptones. Triki-Ellouz *et al.* (2003) reported high protease activity and bacterial growth when fish protein hydrolysate is hydrolyzed by *Pseudomonas aeruginosa* MN7, and Ovissipour *et al.* (2009) revealed that fish protein hydrolysates obtained from yellowfin tuna fisheries by-product, produced with Alcalase which has higher content of small peptides, was more auspicious for the growth of many bacteria, among them *Pseudomonas aeruginosa* and *Bacillus subtilis*. In a recent study performed by Safari *et al.* (2012), also with yellowfin tuna heads, it was also reported that several strains of *Lactobacillus* genus had a higher specific growth rate with fish hydrolysates produced with Alcalase, which also had the highest degree of hydrolysis. The utilization of fish protein hydrolysate of round sardinella as culture media for *Rhizopus oryzae* fungus to produce lipase was studied by Ghorbel *et al.* (2005). In this experiment, a higher production of lipase was achieved with the hydrolysate than a standard culture medium, where the nitrogen source was soya peptone.

CHAPTER II - MATERIALS AND METHODS

1. MATERIALS

The materials used in this study were commercial cod protein hydrolysate (CPH), provided by Danish Fish Protein, Denmark, yeast extract (YE) purchased from Oxoid™ (Oxoid Limited, Basingstoke, UK) and sucrose provided by AnalAR® (VWR International - Material de Laboratório, Lda., Carnaxide, Portugal). Fluorescein isothiocyanate (FITC), *o*-Phthaldialdehyde (OPA), 2,2 – Diphenyl – 1 – picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), pyrocatechol violet and 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) were purchased from Sigma-Aldrich® (St Louis, MO, USA). The acetonitrile and trifluoroacetic acid (TFA) used in the cleaning in place system (CIP) of the FPLC ÄKTA system were purchased from Merck (Merck KGaA, Darmstadt, Germany) All other chemicals were of analytical grade.

2. METHODS

2.1. Hydrolysates production

2.1.1. Preparation of culture media and microbiological assay

Fish protein hydrolysates were prepared by a fermentative process using a proteolytic strain, presumptively a *Bacillus* sp., previously isolated from a clam *Venerupis philippinarum*. Three different culture media were used, containing 0.5 % sucrose (w/v), 0.5 % yeast extract (w/v) and 1 %, 2 % and 4 % CPH (w/v). All culture media were coldly dissolved and sterilized at 121 °C for 15 minutes.

Starter culture of *Bacillus* sp. was plated in Tryptic Soy Agar medium (TSA) and incubated at 30 °C for 24 hours. After that, 250 µL of the inoculum with 10⁴ CFU mL⁻¹ were transferred to an 500 mL-Erlenmeyer flasks containing 250 mL of each sterilized medium and incubated at 30 °C with constant agitation (150 rpm). Incubation periods lasted for 24, 50 and 72 hours, with 0.1 mL of the inoculum being plated in Plate Count Agar (PCA) and placed in an incubator at 30 °C for two days, in order to evaluate the microbial population in the culture medium.

Each assay was made in duplicate.

2.1.2. Optical Density (OD) measurements and separation of the bacteria from the medium

After each incubation period, the OD of the fermentative protein hydrolysate (FPH) was measured in a UNICAM UV – visible UV2 spectrophotometer (Thermo Scientific, Karlsruhe, Germany) at 600 nm in order to evaluate the microbial population. Then, the medium was centrifuged at 10 000 rpm for 5 minutes, at room temperature in a Kubota 6800 centrifuge (Kubota Corp., Tokyo, Japan), to separate the biomass. The supernatant was filtrated in a Millipore filtration system (EMD Millipore Corporation, USA), equipped with a filter with 0.45 µm pore diameter to remove remaining bacteria, and identified as 1 % FPH, 2 % FPH and 4 % FPH.

2.1.3. Supernatant storage, concentration and freeze-drying

An aliquot of the total volume of the supernatant (approximately 5 mL) was withdrawn, in order to determine the enzymatic activity and the peptide profile of the FPH. The remaining volume of the culture medium was concentrated using a rotatory evaporator (Rotavapor RE 121, Büchi Laboratory Equipment, Flawil, Switzerland) equipped with a vacuum pump (V - 700, Büchi Laboratory Equipment, Flawil, Switzerland). The binomial temperature/pressure used was 40 °C at 75 mPa. After evaporating most of the water of the FPH, samples were frozen and lyophilized (Thermo Scientific Heto Power Dry LL 3000, Labosistema, Portugal) for 24 - 28 hours. The resulting powder was stored at – 20 °C until used further use.

2.2. Hydrolysates characterization

2.2.1. Determination of enzymatic activity

The enzymatic activity was determined following the method described by Twining (1984), with slight modifications, using derivatized casein with 1 % (w/v) of FITC as substrate.

The derivatized casein substrate was prepared dissolving 1 g of casein in 100 mL of 50 mM carbonate buffer pH 9.5, containing 150 mM sodium chloride. Then, 40 mg of FITC were

added to the mixture, obtaining an orange solution which was kept under stirring for 3 hours at room temperature. Free FITC was removed by dialyzing four times. The first two were made against a suspension of activated charcoal in water; the next dialysis was done against 50 mM Tris buffer pH 8.5, and finally, against a 50 mM Tris buffer pH 7.2. All dialyses were performed at 4 °C under stirring overnight. The protein concentration was adjusted to 0.5 % (w/v) by adding 50 mM Tris pH 7.2 to a final volume of 200 ml. This substrate was stored in aliquots, frozen at -20 °C until further use.

After each sampling time (24, 50 and 72 hours), 30 µL of Tris buffer 100mM pH 7.8, 10 µL of casein substrate and 10 µL of FPH were mixed in an *Eppendorf* tube, homogenized and incubated at 37 °C for 30 minutes. After the incubation period, 120 µL of trichloroacetic acid (TCA) 5 % (w/v) were added to the samples. The samples were centrifuged at 10 000 rpm, for 5 minutes (Sigma 2K15, B. Braun, Melsungen, Germany), and 100 µL of the supernatant were transferred to a tube with 2.9 mL of tris buffer 500 mM pH 8.5.

In parallel, two controls were also made, where the FPH was replaced by the same volume (10 µL) of Tris buffer 100 mM pH 7.8. In the positive control the 120 µL TCA 5 % (w/v) were replaced by 120 µL Tris buffer 500 mM pH 8.5. In the negative control the same volume of TCA 5 % was added. All samples and controls were homogenized and fluorescence measurements were made in a spectrofluorometer (LS 50B, PerkinElmer Inc, Waltham, Massachusetts USA), using an excitation wavelength of 495 nm and an emission wavelength of 525 nm.

The results regarding the enzymatic activity were expressed in relative fluorescence, calculated by the following equation:

$$\text{Relative fluorescence (\%)} = \frac{F_s - F^-}{F^+ - F^-} \times 100$$

where F_s is fluorescence after the enzymatic reaction, F^- is the fluorescence of the negative control and F^+ is the fluorescence of the positive control. Each measurement was made in quadruplicate.

2.2.2. Gel filtration and peptide profile

In order to characterize the peptide profile of culture medium and the FPH obtained, a gel filtration chromatography was made in a FPLC ÄKTA system (Amersham Biosciences, Uppsala, Sweden) equipped with a UV detector at 280 nm. The column used was a Superdex™ Peptide 10/300 GL (Amersham Biosciences, Uppsala, Sweden).

The molecular weight (MW) calibration curve was prepared using ribonuclease A (13,700 Da), aprotinin (6,500 Da), angiotensin I (1,296 Da), triglycine (189 Da), glycine (75 Da) and blue dextran.

2.2.3. Protein content determination

The protein content was determined using a FP-528 LECO nitrogen analyzer (LECO; St Joseph, MI, USA) calibrated with ethylenediaminetetraacetic acid (EDTA) according to the Dumas method described by Saint-Denis & Goupy (2004). The experiment was performed in triplicate.

2.2.4. Yields of protein recovery

The yield of the different FPH samples was calculated according to the following equation:

$$\text{Yield (\%)} = \frac{w_{\text{FPH}} \cdot P_{\text{FPH}}}{w_{\text{s}} \cdot P_{\text{s}}} \times 100$$

where w_{FPH} is the weight in grams of FPH, P_{FPH} is the protein content (%) of FPH, w_{s} is the weight of substrate in grams and P_{s} is the protein content (%) of substrate.

2.2.5. Degree of hydrolysis (DH)

DH was measured using OPA following the method referred by Nielsen *et al.* (2001). An aliquot of the sample (400 μL) was added to 3 mL of OPA solution and homogenized for 5 seconds. The mixture's absorbance was measured 2 minutes later at 340 nm in a UNICAM UV – visible UV2 spectrophotometer. The same volume of distilled water instead of FPH sample was used as control. In parallel, it was also prepared a serine standard.

DH was also determined for the YE and CPH separately. These determinations were only possible to make for samples of 0 hours of fermentation, since this was the only sampling time in which the proportion of these two components was known.

The DH was expressed in percentage and calculated using the following formulas:

$$DH = \frac{h}{h_{tot}} \times 100$$

$$h = \frac{\text{Serine-NH}_2 - \beta}{\alpha}$$

$$\text{Serine} - \text{NH}_2 = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{control}}}{\text{Abs}_{\text{serine}} - \text{Abs}_{\text{control}}} \times \frac{0.9516 \times 0.1 \times 10}{w \times P}$$

where $\text{Abs}_{\text{sample}}$ is the absorbance of FPH, $\text{Abs}_{\text{serine}}$ is the absorbance of serine standard, $\text{Abs}_{\text{control}}$ is the absorbance of control, w is the weight in grams of FPH sample in 100 mL and P is the protein content (%) of FPH. Constants values of the number of equivalent peptide bonds per gram of protein (h_{tot}), α and β were assumed to be 8.6 meq g^{-1} , 1 and 0.4, respectively, since these are the recommended values for fish by Nielsen *et al.* (2001).

Each experiment was made in quadruplicate.

2.3. Antioxidant properties of FPH

2.3.1. Antiradical activity

DPPH radical scavenging activity

DPPH radical scavenging activity was measured according to the procedure described by Morales & Jiménez-Pérez (2001) and Picot *et al.* (2010), with slight modifications. One milliliter of the sample with different concentrations was added to 1 mL of daily prepared solution of 0.1 mM DPPH in 95 % ethanol. The mixture was homogenized and shaken for 1 hour, at 24 °C. After the incubation period, samples were centrifuged at $10\,000 \times g$ for 10 minutes in a Sigma 2K15 centrifuge, and the absorbance of the resultant supernatant was measured at 517 nm in a UNICAM UV – visible UV2 spectrophotometer. The control sample contained a solution of 0.5 % sucrose (w/v), in order to eliminate the effect of sucrose in radical scavenging activity of the sample.

DPPH inhibition was also determined for the YE and CPH separately. These determinations were only possible to make for samples of 0 hours of fermentation.

Radical scavenging activity of the samples was expressed as the percentage of disappearance of DPPH:

$$\text{Inhibition (\%)} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

where $Abs_{Control}$ is the difference between the absorbance of distilled water and the absorbance of the control sample with 0.5 % sucrose, and Abs_{sample} is the absorbance of the FPH sample.

The DPPH scavenging activity assay was made in quadruplicate and later expressed as EC_{50} , corresponding to the concentration of protein of FPH that is able to scavenge 50 % of DPPH radical.

ABTS radical scavenging activity

ABTS radical scavenging activity was determined using the method described by Re *et al* (1999). To prepare the 7 mM ABTS⁺ solution, 10 mg of ABTS were dissolved in 2.6 mL of a 2.45 mM potassium persulfate solution. The solution remained for 16 hours in the dark at room temperature before use. The ABTS⁺ solution was diluted with 5 mM sodium phosphate buffer pH 7.4, to give an absorbance value of 0.7 ± 0.02 at 734 nm. Then, 20 μ L of sample solutions at different concentrations were added to 2 mL of the diluted ABTS⁺ solution and the mixture was homogenized and incubated at 30 °C for 6 minutes. In parallel, a control sample was prepared with 0.5 % sucrose solution (w/v), instead of the FPH samples. ABTS inhibition was also determined for the YE and CPH separately. The samples absorbances were measured at 734 nm in a UNICAM UV – visible UV2 spectrophotometer.

Radical scavenging activity of the samples was expressed in percentage of inhibition:

$$\text{Inhibition (\%)} = \frac{Abs_{Control} - Abs_{Sample}}{Abs_{Control}} \times 100$$

where $Abs_{Control}$ is the absorbance of the control sample with 0.5 % sucrose, and Abs_{sample} is the absorbance of the FPH.

The results were expressed as Trolox equivalent antioxidant capacity. A standard curve was prepared using Trolox (0.25, 0.5, 1.0 and 1.5 mM) as standard.

ABTS scavenging activity assay was made in quadruplicate.

2.3.2. Reducing Power

The reducing power of culture media and FPH samples was determined according to the method described by Oyaizu (1986). The assay was done by mixing 2 mL of each sample (0.5 % sucrose solution, for the control), 2 mL of 0.2 M phosphate buffer pH 6.6, and 2 mL of 1 % (w/v) potassium ferricyanide. The mixture was homogenized and incubated for 20 minutes at 50 °C. After the incubation period, 2 mL of 10 % (w/v) TCA was added and the mixture was centrifuged at 1 500 x g for 10 minutes in a Sigma 2K15 centrifuge. Then, 2 mL of the supernatant were mixed with 2 mL distilled water and 0.4 mL of 0.1 % ferric chloride. This mixture was incubated for 10 minutes at room temperature and their absorbance was measured at 700 nm in a UNICAM UV – visible UV2 spectrophotometer. Reducing power was also determined for the YE and CPH separately.

The results were expressed as acid ascorbic equivalent reducing power. A standard curve was prepared using ascorbic acid (0.02, 0.04, 0.06, 0.08, 0.10 and 0.12 mg mL⁻¹) as standard.

This assay was made in quadruplicate.

2.3.3. Metal ion chelating activity

Copper

Copper chelating activity was ascertained according to the procedure described by Saiga *et al.* (2003), with some modifications. Solution of 0.1 mg mL⁻¹ copper sulfate in sodium acetate buffer 50 mM pH 6.0 was prepared, as well as a solution of 0.3 mM PV in sodium acetate buffer 50 mM pH 6.0. One milliliter of FPH sample was added to 1 mL of the copper sulfate solution, and the mixture was homogenized. Next, 250 µL of PV 50 mM solution were added and homogenized. Copper chelating activity was also determined for the YE and CPH separately. Absorbance was measured at 626 nm in a UNICAM UV – visible UV2 spectrophotometer.

Chelating activity was calculated using the following formula:

$$\text{Chelating activity (\%)} = \frac{\text{Abs (PV+Cu}^{++}) - \text{Abs (PV+ Cu}^{++}\text{+sample)}}{\text{Abs (PV+ Cu}^{++})} \times 100$$

where Abs (PV + Cu⁺⁺) is the absorbance of the PV + Cu⁺⁺ complex in the absence of peptide samples and Abs (PV + Cu⁺⁺ + sample) is the absorbance of the PV + Cu⁺⁺ complex in the presence of peptide samples.

This assay was made in quadruplicate.

Iron

The chelating activity for iron was determined in the same way as mentioned by Decker & Welch (1990). One milliliter of samples with different concentrations was mixed with 3.7 mL of distilled water and 100 µL of ferrous chloride 2 mM, followed by homogenization. Then, 200 µL of ferrozine 5 mM was added to the mixture and it was shaken vigorously. Ten minutes later, the samples absorbance was measured at 565 nm in a UNICAM UV – visible UV2 spectrophotometer. Iron chelating activity was also determined for the YE and CPH separately for 0 hours of hydrolysis, since this was the only sampling time in which the proportion of these two components was known.

Chelating activity was estimated using the equation below:

$$\text{Chelating Activity (\%)} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

where Abs_{Control} is the absorbance of the control sample with 0.5 % sucrose and Abs_{sample} is the absorbance of the FPH.

This assay was made in quadruplicate.

2.4. Statistical analysis

A factorial ANOVA was applied to determine significant differences between the FPH samples ($p \leq 0.05$). Multiple comparisons were done by the Tukey HSD test. In the absence of homogeneity/normality conditions, a Kruskal – Wallis methodology was applied for the multiple comparisons. Statistical treatment was done with software STATISTICA® from StatSoft Inc. (Tulsa, OK, USA), version 7.0, 2004.

CHAPTER III - RESULTS AND DISCUSSION

1. MICROBIAL GROWTH AND PROTEASE PRODUCTION

The evolution of bacterial growth was evaluated by OD and total counts of colony-forming units (CFU) in the three experiments with 1, 2 and 4 % of CPH in figure 3.

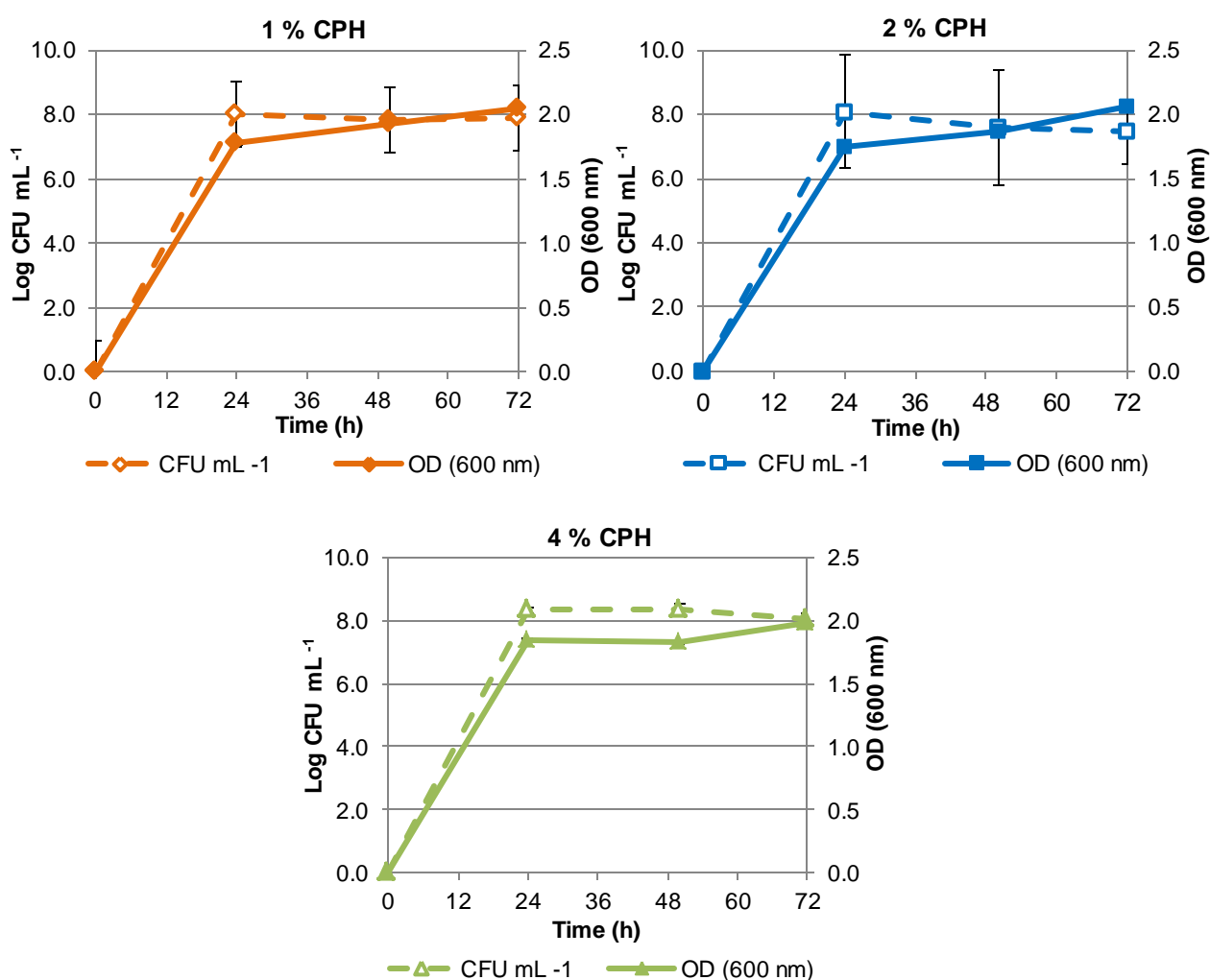


Figure 3. Growth of *Bacillus* sp. strain in three culture media.

As it can be seen, a fast bacterial growth was recorded in all experiments after 24 hours and remained practically constant in the next two days. These results suggest an exponential growth until 24 hours, followed by a stationary phase afterwards.

The maximum of proteolytic activity was recorded 24 hours after the beginning of the assay for all culture media, when the strain entered in its late exponential or early stationary phase of bacterial growth (fig. 4). The production of proteases in this period by bacteria has been described by many authors as a common characteristic in many bacterial strains (Razak *et al.*, 1997; Shivanand *et al.*, 2009; Uyar *et al.*, 2011). Sousa *et al.* (2007) also obtained a maximum of enzymatic activity after 24 hours of incubation, followed by a gradual decrease of proteolytic activity. In this study it was also verified a correlation between the growth of *Bacillus cereus* and the protease production, indicating that secretion of proteases is related with the bacterial growth or with stationary phase, with greatest expression of protease during the stationary phase, when sporulation also occurs.

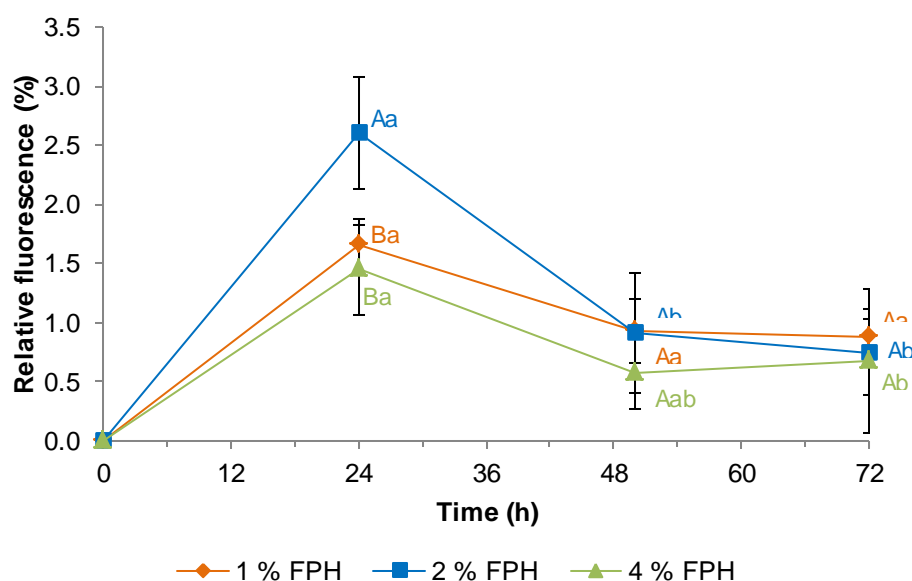


Figure 4. Effect of CPH concentration in the protease production. The same letter indicates values not significantly different ($p > 0.05$), with A, B, C comparing the same fermentation time and a, b, c comparing the same type of culture medium.

After 24 hours, the decline of protease activity recorded in all experiments suggests that somehow optimal conditions weren't reunited during the assay. Sousa *et al.* (2007) reported that bacteria of the *Bacillus* genus secrete extracellular metalloproteases which require metal ions, like Ca^{2+} , in order to reach its optimal activity. The lack of Ca^{2+} in the culture medium justifies the decrease of proteolytic activity in the assays, since the enzyme

produced requires this metal ion for their catalytic mechanism (data not shown). Other explanation for the decline of protease activity resides in the limitation of the Twinning method (1984), which doesn't involve Ca^{2+} in the measurements, leading to a misreading of the real fluorescence value.

The amount of nitrogen source in the media didn't seem to affect significantly bacterial growth, but the same didn't happen with the enzyme production. The differences recorded in the enzymatic activity within the three assays may be related with the different C/N ratio among them (fig. 4). In fact, Beg *et al.* (2002) demonstrated that the C/N ratio strongly influences the production of extracellular proteases. In this case, it seems that the medium with 2 % CPH had an adequate C/N ratio for the enzyme production, while 4 % CPH medium contained a very high concentration of nitrogen.

2. EVOLUTION OF PROTEIN CONTENT DURING BACTERIAL GROWTH AND DEGREE OF HYDROLYSIS

The evolution of protein content of the different FPH samples was evaluated by the Dumas methodology. Table 2 indicates the protein content in percentage of dry weight (DW) for all experiments.

Table 2. Evolution of protein content (% DW) of FPH samples, as well as commercial CPH and YE used in their formulations. Means with the same letters indicates values not significantly different ($p > 0.05$), with A, B, C comparing the same fermentation time and a, b, c, d comparing the same FPH sample.

Sample	Time (hours)			
	0	24	50	72
1 % FPH	52.06 ± 0.30 ^{Ca}	49.95 ± 0.02 ^{Cb}	43.23 ± 0.05 ^{Cc}	39.91 ± 0.05 ^{Cd}
2 % FPH	56.70 ± 0.33 ^{Bb}	58.63 ± 0.14 ^{Ba}	55.01 ± 0.21 ^{Bc}	49.58 ± 0.24 ^{Bd}
4 % FPH	63.15 ± 0.30 ^{Ab}	65.55 ± 0.25 ^{Aa}	63.19 ± 0.09 ^{Ab}	61.64 ± 0.12 ^{Ac}
CPH	72.51 ± 0.09 *	-	-	-
YE	67.34 ± 0.11 *	-	-	-

*Not included in statistical analysis.

It is possible to verify a general decrease of protein content of all samples with the increase of fermentation time. As it was expected, the 4 % FPH presented the highest percentage of protein in DW, followed by the 2 % FPH sample.

The general decrease of protein content of FPH with the fermentation time may be due to the higher consumption of nitrogenous compounds than sucrose by the bacteria, which led to higher percentage of this component particularly after 72 hours.

The yields obtained in this process based on the protein content (fig. 5) presented a significant decrease ($p \leq 0.05$). They reflect the general decrease of protein content of FPH.

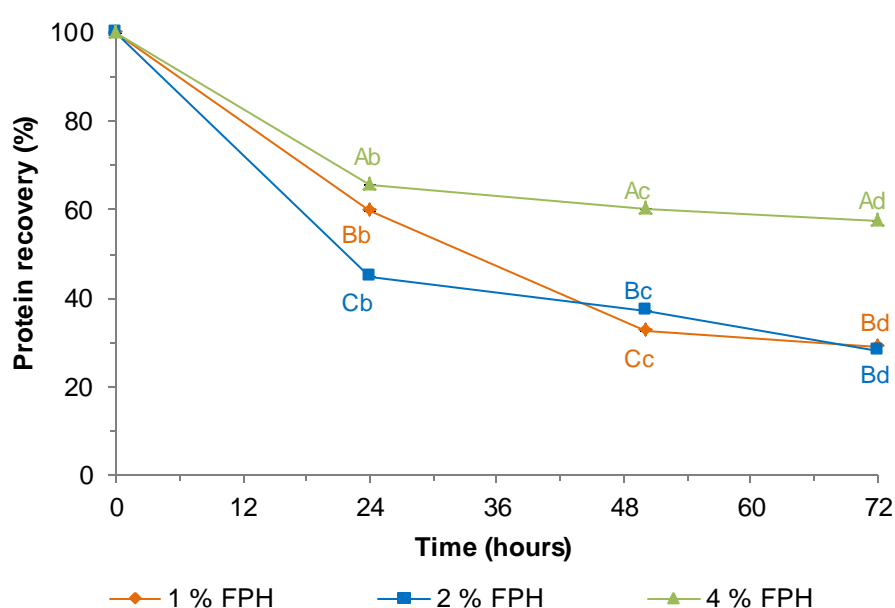


Figure 5. . Evolution of protein recovery in all FPH samples. The same letter indicates values not significantly different ($p > 0.05$), with A, B, C comparing the same fermentation time and a, b, c, d comparing the same type of culture medium

The extent of protein hydrolysis by the *Bacillus* sp. strain was measured by assessing the degree of hydrolysis (DH), which also allows a deeper understanding of the evolution of the biological properties of the newly formed hydrolysates. In table 3 is shown the evolution of the DH during the fermentation process, as well as the DH of commercial CPH used.

Table 3. DH (%) values of FPH samples. Means with the same letters indicates values not significantly different ($p > 0.05$), with A, B, C comparing the same fermentation period and a, b, c, d comparing the same FPH sample.

Sample	Time (hours)			
	0	24	50	72
1 % FPH	26.13 \pm 0.54 ^{Aa}	20.92 \pm 0.37 ^{Ac}	23.68 \pm 0.63 ^{Ab}	23.34 \pm 0.41 ^{Bb}
2 % FPH	20.49 \pm 0.39 ^{Bc}	19.37 \pm 0.22 ^{Bd}	22.16 \pm 0.54 ^{Bb}	26.76 \pm 0.24 ^{Aa}
4 % FPH	17.21 \pm 0.12 ^{Cbc}	16.29 \pm 0.24 ^{Cc}	18.11 \pm 0.59 ^{Cb}	19.36 \pm 0.44 ^{Ca}
CPH	13.52 \pm 0.21 *	-	-	-

*Not included in statistical analysis.

A significant increase ($p \leq 0.05$) of DH recorded in 2 % and 4 % FPH obtained after 72 hours was observed. In the case of 1 % FPH this increase of DH during the first 24 hours of the fermentative process was not recorded. The high initial DH was due to the YE which has compounds reacting with OPA reagent and thus contributing to the high absorbance measured giving an overestimated DH. This led to an apparent decrease of DH calculated in 1 % FPH. This effect was also certainly present in the other FPH, but was not so evident since they had a lower proportion of YE.

3. MOLECULAR WEIGHT DISTRIBUTION

The MW distribution of hydrolysates obtained was very similar for the three experiments. In figure 6 is shown a gel filtration chromatogram of all samples after 72 hours. As it can be seen there are an equal number of peaks in the 1 % and 2 % FPH samples (five peaks); while the medium with 4 % FPH has one less peak (four peaks). In figure 6 it is also evident that for all FPH the first three peaks present the same elution volume, i.e., they correspond to hydrolysates with similar molecular weight. The first two peaks coincide to peptides with MW higher than 400 Da (peak ①) and peptides between 200 and 75 Da (peak ②). The major differences occur after 20 mL of elution, which corresponds to the class of hydrolysates with MW equal or lower than 75 Da. Although there are no peptides or amino acids with MW lower than 75 Da, these results may be explained by interactions between the column and the smaller peptides and amino acids, increasing their elution volume and giving underestimated MW values. Nevertheless, it's in this part of the chromatogram that are

represented the variety of smaller-molecular-weight peptides in each FPH sample, which might influence their antioxidant properties.

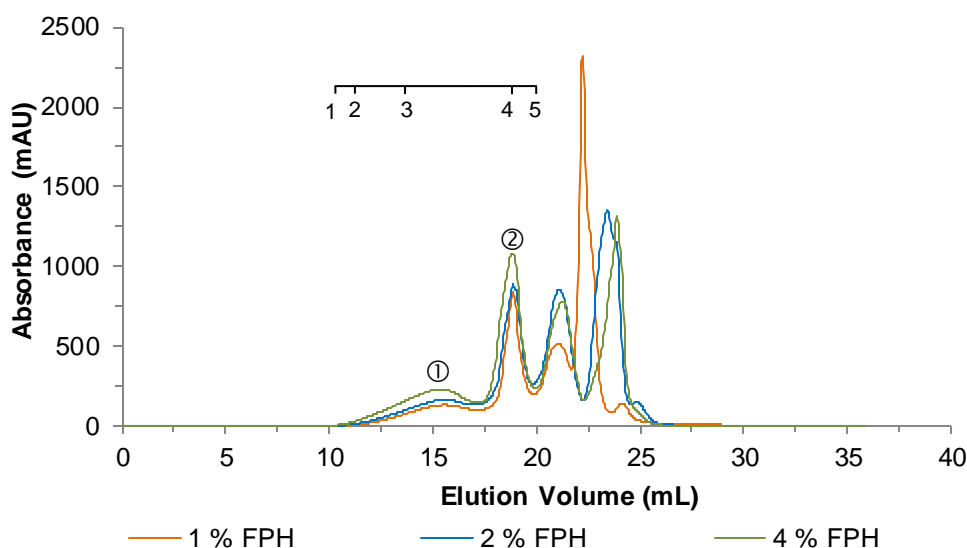


Figure 6. Gel filtration chromatogram showing the molecular weight distribution of hydrolysates at 72 hours of hydrolysis. Numbers represent the peaks' MW classes: 1 corresponds to ribonuclease A (13 700 Da), 2 corresponds to aprotinin (6 500Da), 3 corresponds to angiotensin I (1 296 Da), 4 corresponds to triglycine (189 Da) and 5 corresponds to glycine (75 Da). ① and ② represent the peaks corresponding to MW higher than 400 Da and between 200 and 75 Da, respectively.

In figure 7 are shown the fraction of hydrolysates for each FPH sample. As it was expected, the peptide profile clearly changed over time, indicating the hydrolysis of the initial peptides into smaller-molecular-weight peptides. The gel filtration profile of all FPH samples showed the disappearance of peptides with molecular weight between 400 and 200 Da in the first 24 hours of the assay. In figure 7 it is also visible a decrease in fractions with a MW higher than 400 Da in all samples, resulting in a low proportion of peptides (between 10 % and 20 %) with that molecular size after 72 hours. Also, all FPH samples showed a reduction in the fraction of hydrolysates with MW between 200 and 75 Da, setting a ratio between 20 % and 30 % at 72 hours of hydrolysis. At the same time, higher proportions of peptides with MW lower than 75 Da were obtained during hydrolysis time for all experiments. Small-molecular-weight peptides were present in the raw material used in the fermentation process and represented about 30 % of total peptides. However, in samples with 1 % and 2 % FPH the percentage of these peptides reached values higher than 60 % at 72 hours, which means

that at the end of the assay more than half of the FPH was possibly formed by tripeptides, dipeptides and free amino acids.

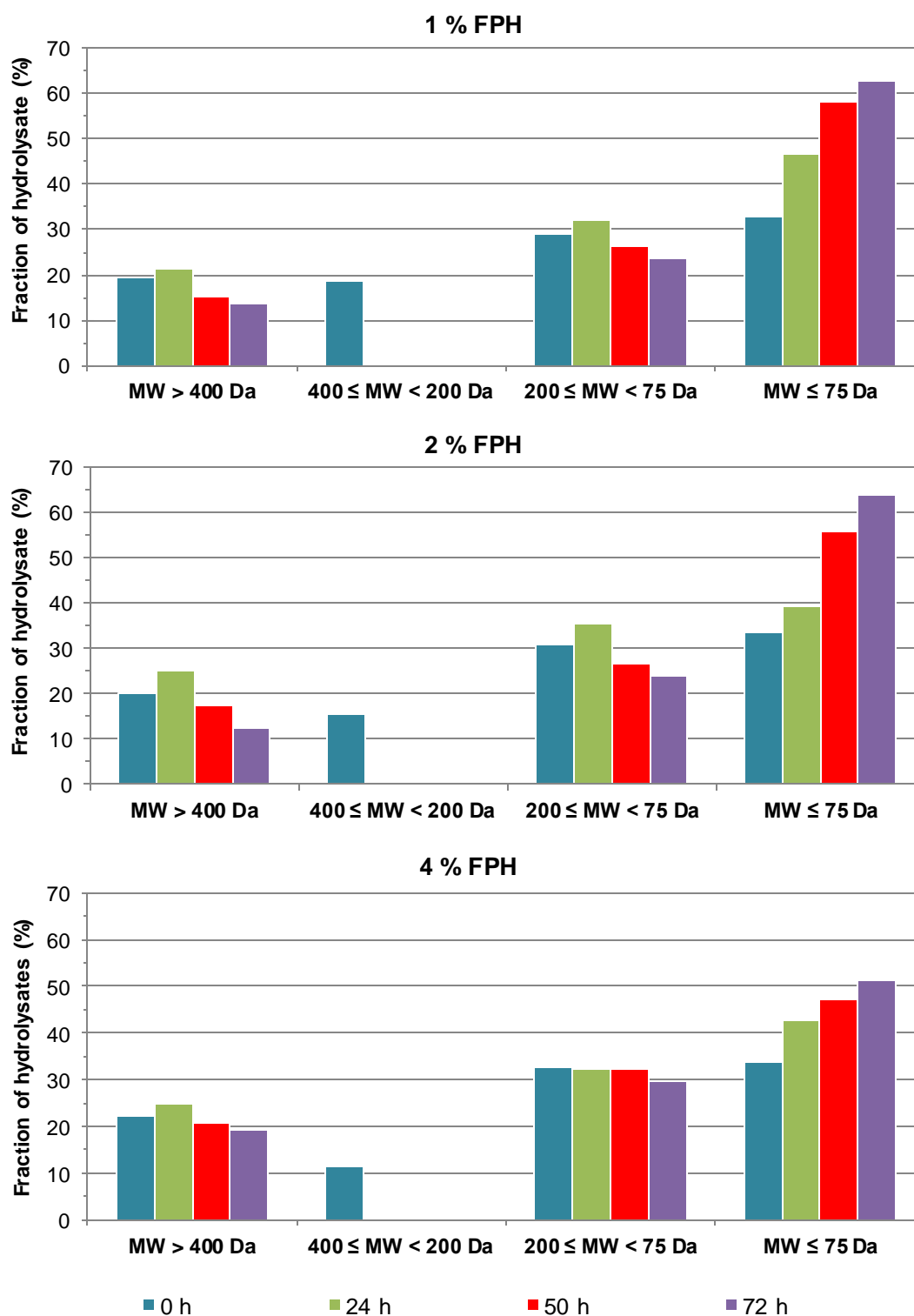


Figure 7. Fractions of the FPH samples according to their molecular weight range for different times of fermentation, 0 hours (blue), 24 hours (green), 50 hours (red) and 72 hours (purple). SD values are not represented, since gel filtration was only performed once for each FPH.

Figure 7 also demonstrates that the concentration of CPH at the initial time influenced the evolution of fraction of hydrolysates in the FPH samples. This is more evident in the 4 % FPH, where the decrease of fraction of hydrolysates with more than 400 Da and between 200 and 75 Da was not so marked as in 1 % or 2 % FPH. Similarly, the proportion of smallest peptides ($MW \leq 75$) was lower than the other two hydrolysates, barely representing 50 % of total peptides after 72 hours of fermentation.

4. ANTIOXIDANT ACTIVITY OF THE FPH

Antioxidants can act by different mechanisms, so it is essential to use several methods for the evaluation of the antioxidant activity (Frankel & Meyer, 2000). In this study DPPH and ABTS radical scavenging activity, reducing power and iron and copper chelating measurements were applied.

4.1. DPPH radical scavenging activity

DPPH assay was used to determine the antiradical activity of commercial CPH, yeast extract (YE) and FPH prepared with 1 %, 2 % and 4 % FPH at the initial time. Figure 8 shows the radical scavenging activity of the samples as a function of the protein concentration.

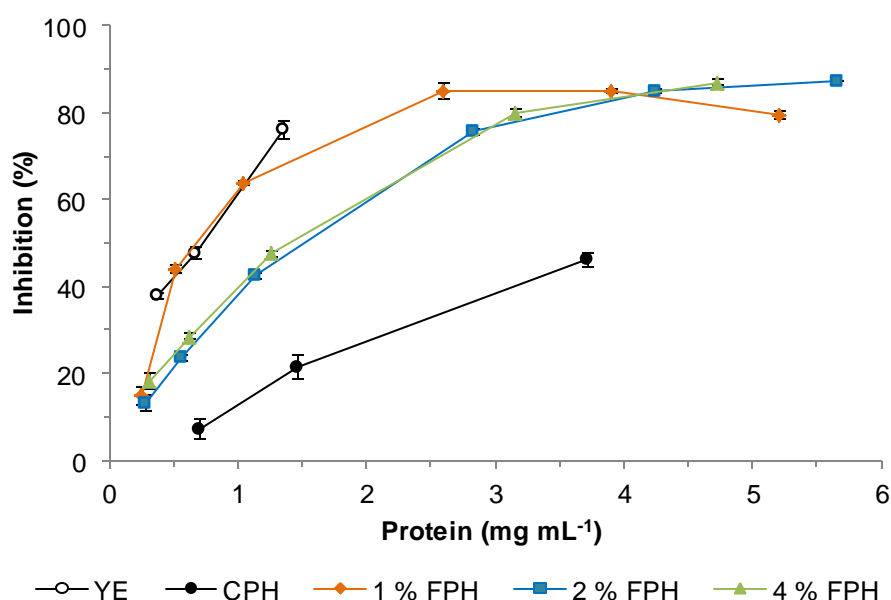


Figure 8. DPPH radical scavenging activity of 1 %, 2 % and 4 % FPH at the initial time (0 hours), CPH and YE.

As it can be seen in figure 8, the YE exhibited high scavenging activity. On the other hand, commercial CPH showed the lowest activity within these five samples. The radical scavenging activity of 1 % FPH reflected the high antiradical activity of YE, where the highest proportion of YE/CPH (1:2) is present. The other samples prepared with 2 % and 4 % of CPH whose proportions of YE/CPH were 1:4 and 1:8 respectively, showed intermediate scavenging activities.

In figure 9 is shown DPPH radical scavenging activity of 1 % FPH obtained after different fermentation times, as a function of protein concentration. The FPH obtained after 24 hours recorded the lowest DPPH antiradical activity, which may be caused by the YE consumption during this period. However, FPH obtained after 50 and 72 hours showed increasing antiradical activity, indicating the formation of peptides with higher radical scavenging activity.

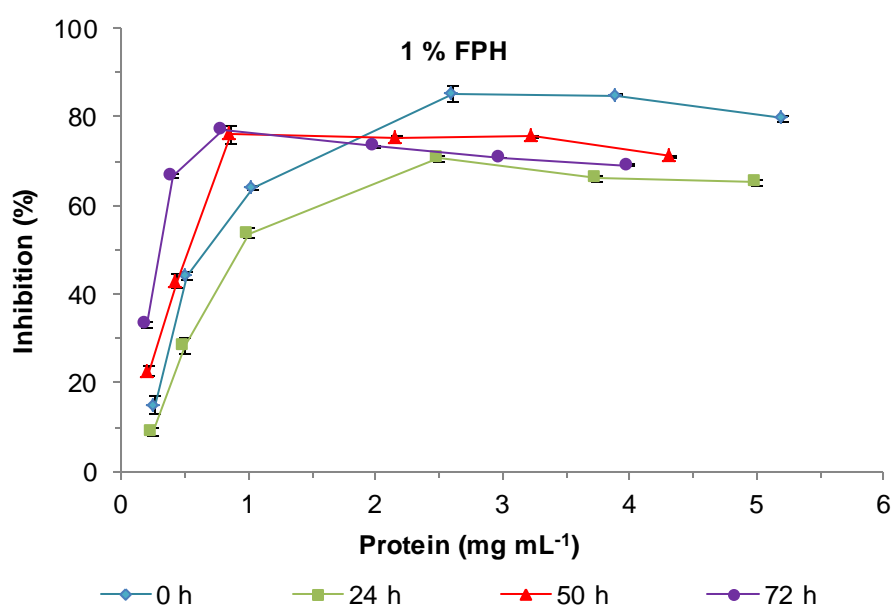


Figure 9. DPPH inhibition of 1 % FPH samples at different times of fermentation.

At the initial time and after 24 hours 2 % FPH seems to exhibit the similar DPPH antiradical activity than 1 % FPH, which may be due to the lower YE/CPH proportion (fig. 10). Samples obtained after 50 and 72 hours of fermentation showed increasing antiradical activity with the increase of fermentation time such as also observed in samples of 1 % FPH.

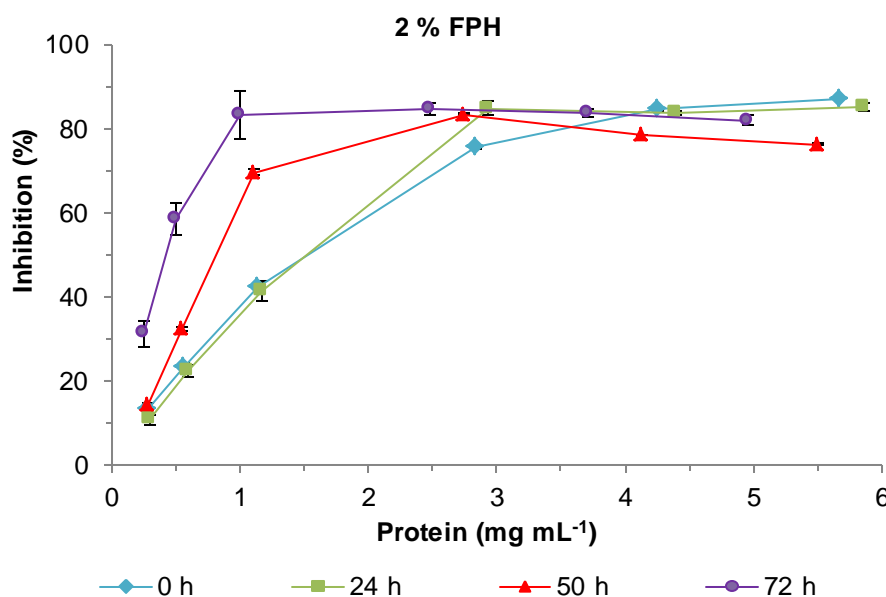


Figure 10. DPPH inhibition of 2 % FPH samples at different times of fermentation.

In the case of 4 % FPH (fig. 11), the hydrolysates prepared after 24 and 50 hours showed relatively low DPPH scavenging activity. However, no plausible explanation was found for these results. On the other hand, there was an increase of antiradical activity after 72 hours of fermentation, although its EC_{50} was lower than those of the 1 % and 2 % FPH obtained after the same fermentation period. This result may be due to some dilution of the new peptides with high radical scavenging activity in a matrix of intact peptides with lower antioxidant activity.

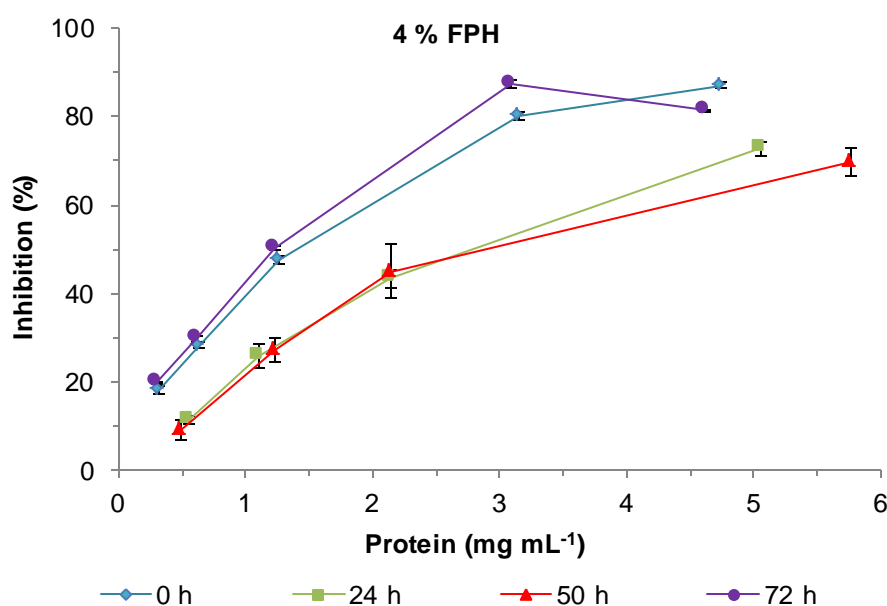


Figure 11. DPPH inhibition of 4 % FPH samples at different times of fermentation.

In order to compare the antiradical activity of FPH, the values of EC_{50} calculated for all samples are presented in figure 12. It can be observed that 1 % and 2 % FPH presented the lowest EC_{50} values, i.e., higher scavenging activity, and evidenced in both FPH the increased formation of antiradical peptides during fermentation time. On the other hand, the lower antiradical activity of 2 % FPH, comparatively to 1 % FPH, until 50 hours of fermentation may result from some dilution of peptides exhibiting antiradical activity in a matrix with higher proportion of non-hydrolyzed peptides. However, no significant ($p > 0.05$) difference in EC_{50} value of this FPH was recorded after 72 hours of fermentation. It was also observed that YE had a considerably high DPPH scavenging activity, which is responsible for the highest initial DPPH scavenging activity of 1 % FPH (that had the highest proportion of YE). However, this effect of YE may have decreased during the fermentation time, which could be responsible for the lower DPPH antiradical activity of 1 % FPH after 24 hours. After this fermentation period, the effect of YE could be diminished and the scavenging activity was mainly due to peptides exhibiting this activity.

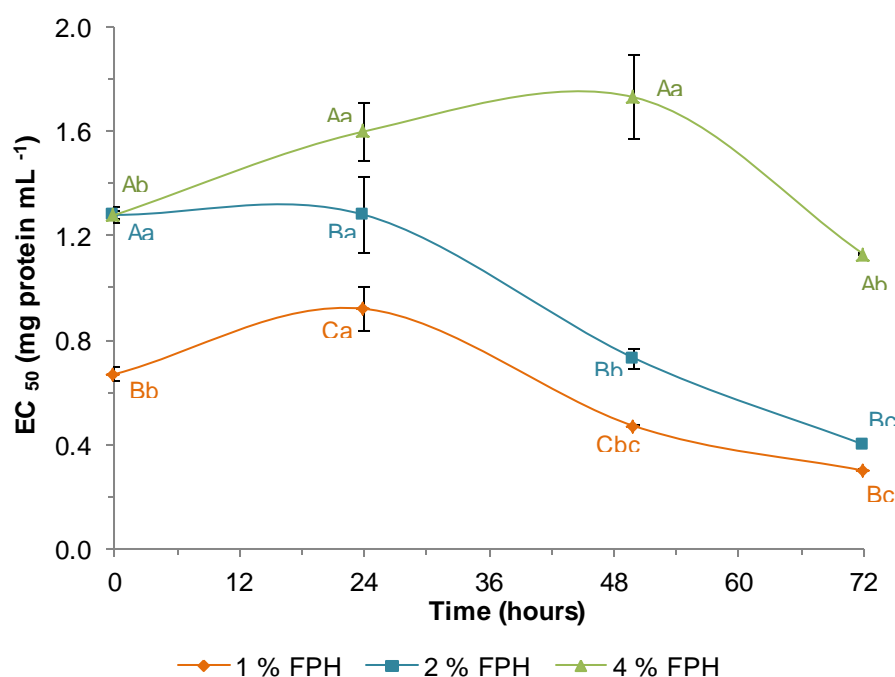


Figure 12. Time course of EC_{50} values of FPH samples. SD values were obtained by four combinations of absorbance values recorded during the DPPH test. The same letter indicates values not significantly different ($p > 0.05$), with A, B, C comparing the same fermentation time and a, b, c comparing the same FPH sample.

This increase of antiradical activity with the fermentation time was not so evident in the case of 4 % FPH, where only a slight non-significant ($p > 0.05$) decrease of this activity was observed in this hydrolysate obtained after 72 hours. This result may also be a consequence of the dilution of the antioxidant peptides formed in a matrix of non-hydrolyzed peptides.

It may be generally concluded that DPPH antiradical scavenging activity of FPH increased with the increase of DH, which was also reported by several authors (Je *et al.*, 2007; Yang *et al.*, 2008; Centenaro *et al.*, 2010). However, the different trends of antioxidant activity of FPH samples rely not only on the length of the peptide chain, but also on the exposed terminal amine groups of the obtained products (Wu *et al.*, 2003; Thiansilakul *et al.*, 2006).

4.2. ABTS radical scavenging activity

The ability to scavenge ABTS radicals was also determined for CPH, YE and FPH samples at the initial time as a function of protein concentration (fig. 13), expressed in Trolox Equivalent Antioxidant Activity (TEAC).

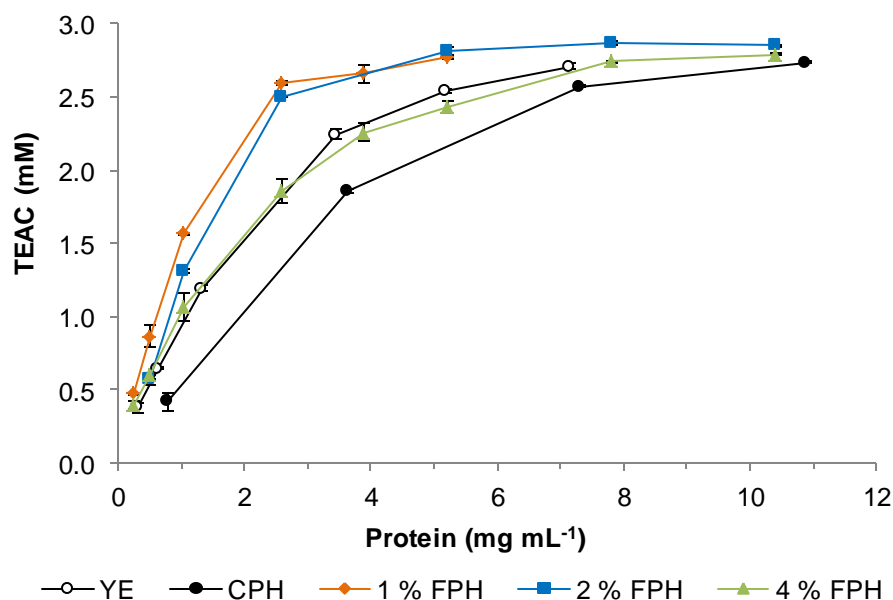


Figure 13. ABTS radical scavenging activity of 1 %, 2 % and 4 % FPH at the initial time (0 hours), CPH and YE.

As it is shown in figure 13, YE exhibited higher ABTS scavenging activity than the commercial CPH. On the other hand, 1 % FPH and 2 % FPH have the best antiradical activity, while 4 % FPH has similar results to the YE, leading to a conclusion that antioxidant capacity of FPH increases with higher proportions of YE/CPH.

In figure 14 are presented the ABTS radical scavenging activity for 1 % FPH, as function of protein concentration obtained after different fermentation periods. It can be observed that antiradical activity presented the same trend for the different periods of fermentation. Nevertheless, this activity after 24 hours reached its lowest value, which may be due to the consumption of YE during this period. On the other hand, at 50 and 72 hours of fermentation 1 % FPH presented an increasing scavenging activity, which may indicate the formation of new peptides capable of eliminating free radical from the ABTS⁺.

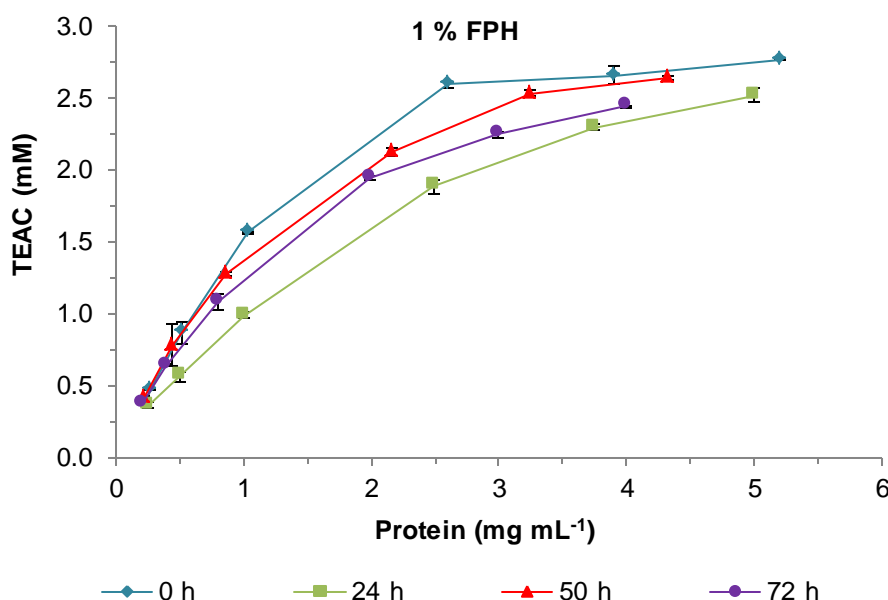


Figure 14. TEAC of 1 % FPH at different times of fermentation.

The results of the 2 % FPH (fig. 15) show a similar decrease of antiradical activity, as in 1 % FPH. However, the decrease of antiradical activity after 24 hours of fermentation does not seem to be as notorious as in the case of 1 % FPH, which might have been caused by the lower proportion of YE/CPH at time zero.

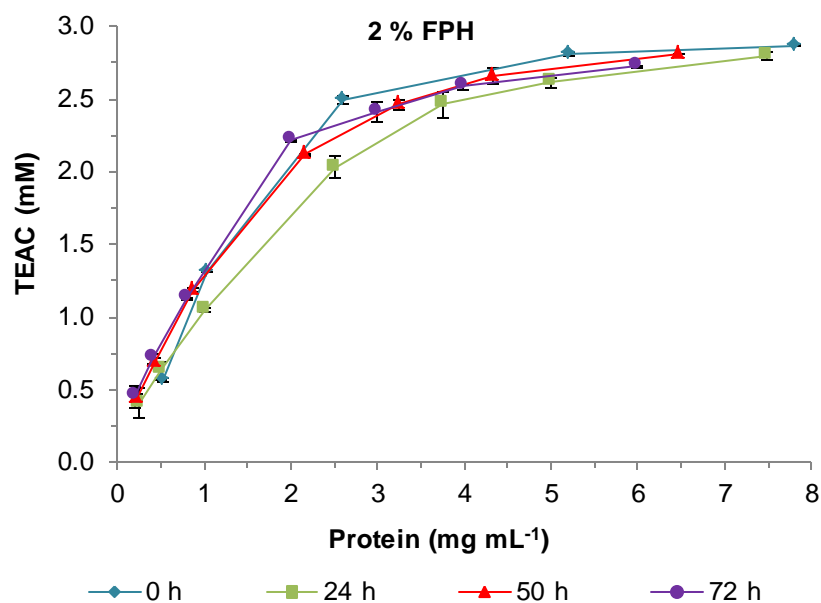


Figure 15. TEAC of 2 % FPH at different times of fermentation.

Contrary to what happened in the previous samples, in 4 % FPH (fig. 16) the decrease of antiradical activity after 24 hours of fermentation was not observed. This similar activity of the initial FPH and that obtained after 24 hours may be justified by the low YE/CPH ratio. The increase of antioxidant activity after 50 and 72 hours, compared to the time zero, indicated the formation of new peptides exhibiting this antiradical activity.

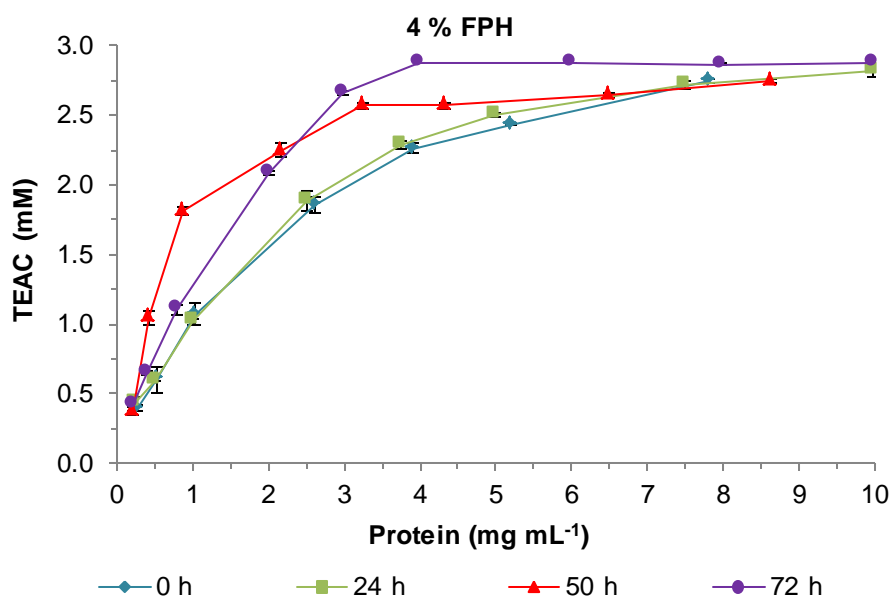


Figure 16. TEAC of 4 % FPH at different times of fermentation.

In order to compare ABTS scavenging activity of all FPH, a concentration of 5 mg FPH mL⁻¹ was chosen and the results were presented in TEAC as a function of fermentation period (fig. 17).

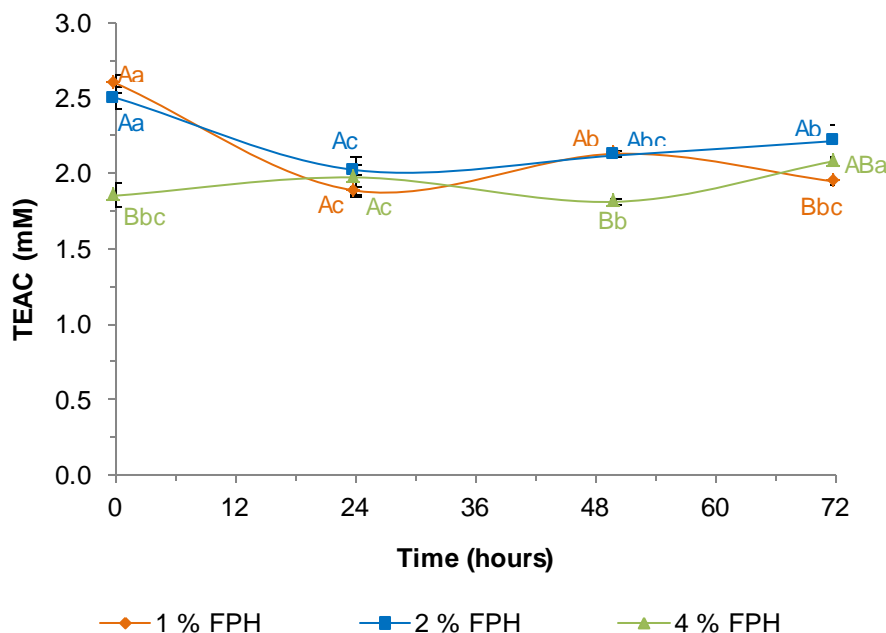


Figure 17. Evolution of ABTS radical scavenging activity for all samples at 5 mg FPH mL⁻¹. The same letter indicates values not significantly different ($p > 0.05$), with A, B, C comparing the same fermentation time and a, b, c, d comparing the same FPH sample.

As it can be observed, there isn't a large variation between the initial and final values of ABTS radical scavenging activity, although they are significantly different ($p \leq 0.05$) in all FPH. At the end of fermentation time, 2 % FPH was the sample which presented better antiradical activity. This hydrolysate obtained after 72 hours presented a significantly higher value ($p \leq 0.05$) than 1 % FPH prepared after the same fermentation period. It must also be emphasized that 4 % FPH was the only sample whose ABTS radical scavenging activity value obtained after 72 hours of fermentation was higher than at the initial time.

The different evolutions of DPPH and ABTS values suggest that these determinations reflect differences in the hydrolysates involved in this radical action. This may be attributed to hydrophilic and lipophilic properties of FPH. Kim *et al.* (2002) referred that these properties are relevant because the ABTS assay is applicable in aqueous and organic phases (i.e.

hydrophilic and lipophilic antioxidant systems), whereas DPPH assay is applicable in organic media. For this reason, ABTS allows a more realistic evaluation of total antiradical.

4.3. Reducing Power

The capacity of the FPH to reduce iron (III) to iron (II) was also evaluated. In figure 18 are represented the results expressed as acid ascorbic equivalent obtained for the different FPH, as well as YE and commercial CPH at the initial time.

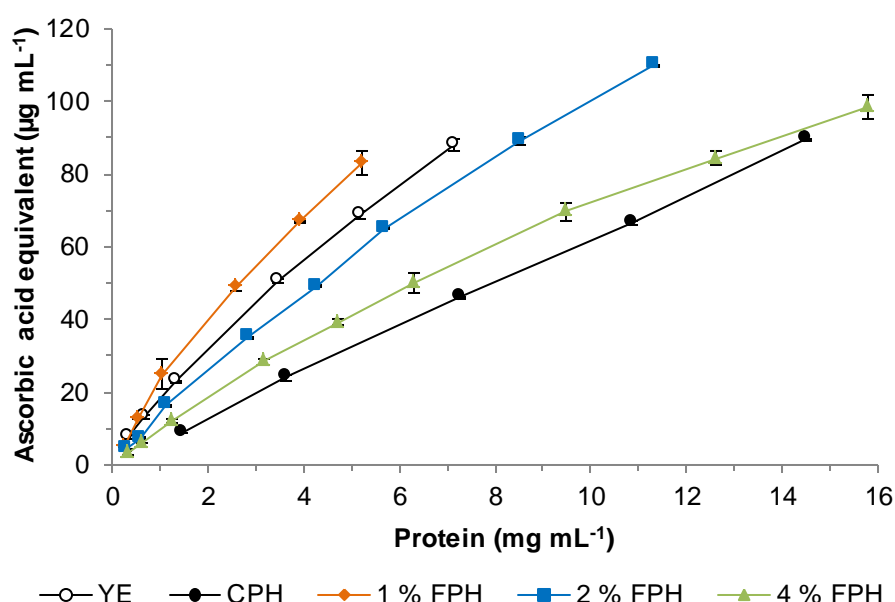


Figure 18. Ascorbic acid equivalent reducing power of 1 %, 2 % and 4 % FPH, CPH and YE at the initial time (0 hours) as a function of protein concentration.

It can be observed that for all FPH there is a linear increase of reducing power with the protein concentration. The YE showed high reducing power, while commercial CPH showed the lowest value within these five samples. A synergistic effect of reducing power between the CPH and the YE constituents seemed to occur. Thus, for one concentration of 1 % FPH it was obtained a positive difference between its measured reducing power (RP_m) and the calculated value of this activity, taking into consideration the individual contributions of CPH and YE ($RP_{YE} + RP_{CPH}$), i.e., $\Delta = RP_m - (RP_{YE} + RP_{CPH})$.

The differences of reducing power among the FPH samples at the initial time of fermentation can be justified by the YE/CPH proportion, once an increasing reducing power

was obtained with the increase of YE/CPH ratio. In fact, this effect of the YE is more evident in 1 % FPH, while the reducing power values of 4 % FPH are closer to those of CPH.

In figure 19 is presented the reducing power of 1 % FPH at different fermentation periods as a function of protein concentration. As it can be seen, there is a decrease of reducing power between the initial time and 24 hours fermentation, which may be due to the YE consumption. Nevertheless, after 50 and 72 hours FPH showed increasing reducing power, which indicates the formation of peptides with better reducing power.

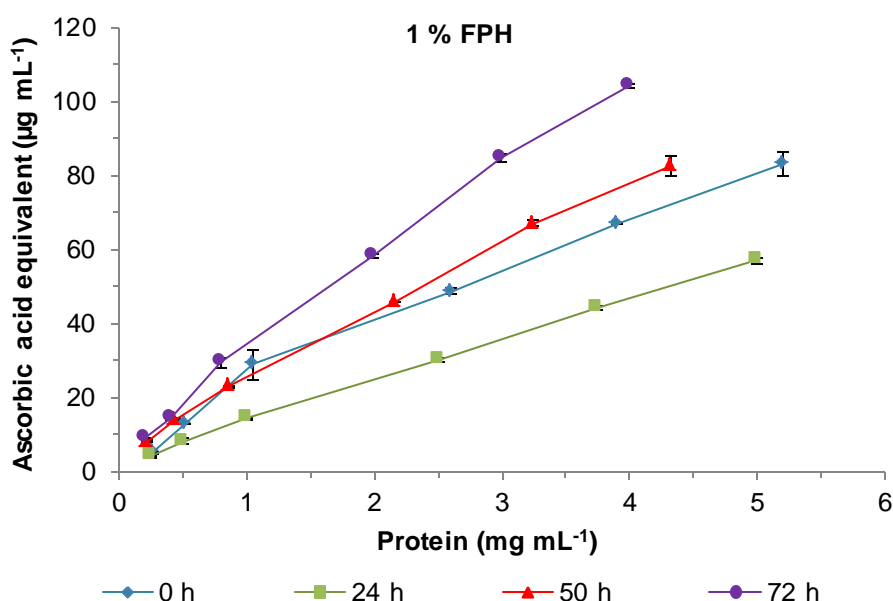


Figure 19. Ascorbic acid equivalent reducing power of 1 % FPH at different times of fermentation.

The results of 2 % FPH (fig. 20) show a similar trend of reducing power with protein concentration, i.e. a decrease of this activity after 24 hours and an increase after 50 and 72 hours of fermentation. However, the increase of reducing power of 2 % FPH obtained after 72 hours seems more notorious as in the case of 1 % FPH.

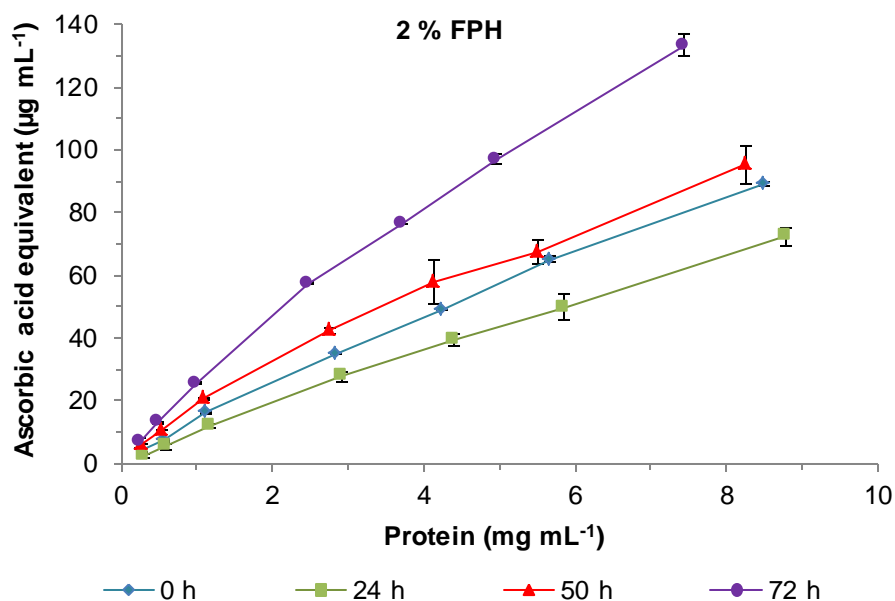


Figure 20. . Ascorbic acid equivalent reducing power of 2 % FPH at different times of fermentation.

For 4 % FPH (fig. 21), similar trends of reducing power were obtained for the FPH prepared after different periods. However, the differences between them were lower than those for 1 % and 2 % FPH. For instance, considering a concentration of protein of 4 mg mL⁻¹ the difference between the hydrolysates obtained after 24 hours and 72 hours was in the range of 43 – 105 µg mL⁻¹ ascorbic acid for 1 % FPH, 35 – 80 µg mL⁻¹ ascorbic acid for 2 % FPH and 35 – 45 µg mL⁻¹ ascorbic acid for 4 % FPH.

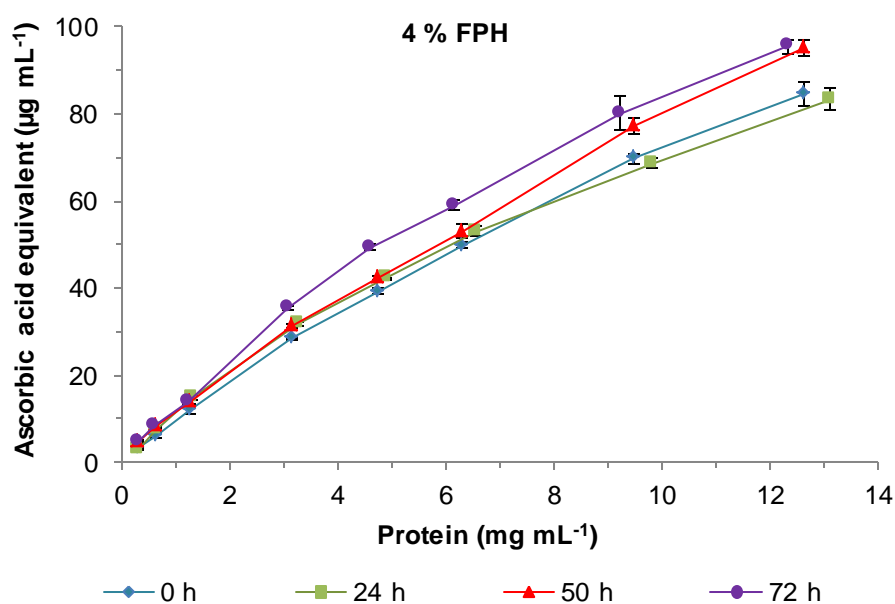


Figure 21. Ascorbic acid equivalent reducing power of 4 % FPH at different times of fermentation.

It should also be referred that the reducing power of all FPH increased with the concentration of protein for all fermentation periods. A similar trend was reported in previous studies (Ktari *et al.*, 2012, Pires *et al.*, 2012).

In order to compare the reducing power of the three FPH as a function of the fermentation period, a concentration of 10 mg FPH mL⁻¹ was chosen and the results are presented in figure 22.

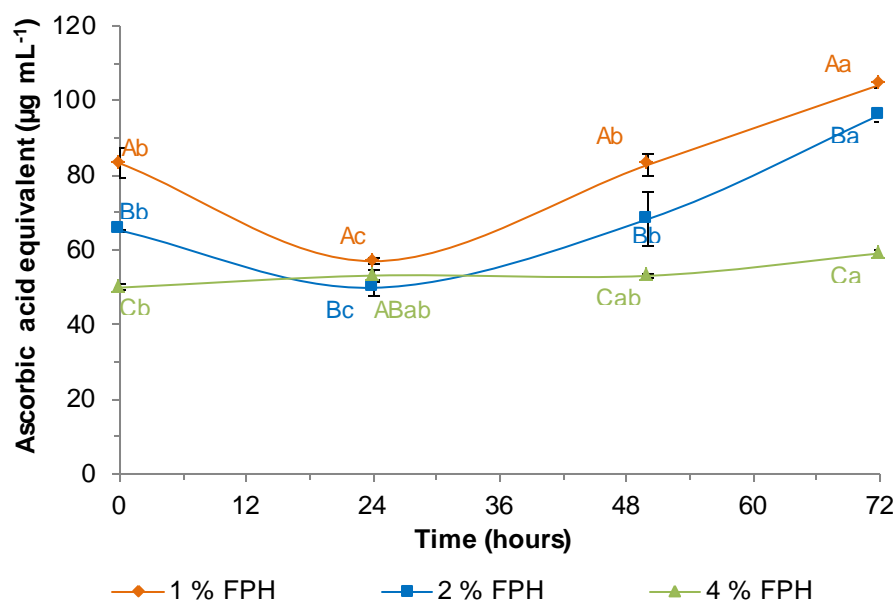


Figure 22. Evolution of reducing power for all samples at 10 mg FPH mL⁻¹. The same letter indicates values not significantly different ($p > 0.05$), with A, B, C comparing the same fermentation time and a, b, c, d comparing the same FPH sample.

As it can be seen, reducing power increased significantly ($p \leq 0.05$) from initial time to 72 hours of fermentation in all FPH. It is also noticeable the decrease of this capacity after 24 hours in 1 % and 2 % FPH, which was probably caused by the disappearance of YE during the first 24 hours of fermentation, while the increase observed at 50 and 72 hours was due to the formation of new hydrolysates with improved reducing power, as it has been previously mentioned.

The increase of reducing power recorded in 4 % FPH after 72 hours was lower than those achieved in 1 % and 2 % FPH. This may be a result of the dilution of the new peptides formed in the intact hydrolysates. It must also be noted that for 4 % FPH it wasn't recorded a decrease of reducing power after 24 hours, which may be due to the lowest YE/CPH ratio preventing a reduction of this capacity by the disappearance of the YE.

According to Je *et al.* (2009) and Pires *et al.* (2012), higher reducing power can be attributed to the high content of peptides that are electron or hydrogen donors that can react with free radicals.

4.4. Copper chelating activity

The capacity to bind transition metals, like Cu^{2+} , is a useful indication of antioxidant activity, because this cation is a catalyst involved in the production of very active radicals resulting from the decomposition of hydrogen peroxide. In figure 23 is presented the Cu^{2+} binding capacity of commercial CPH, YE and FPH samples

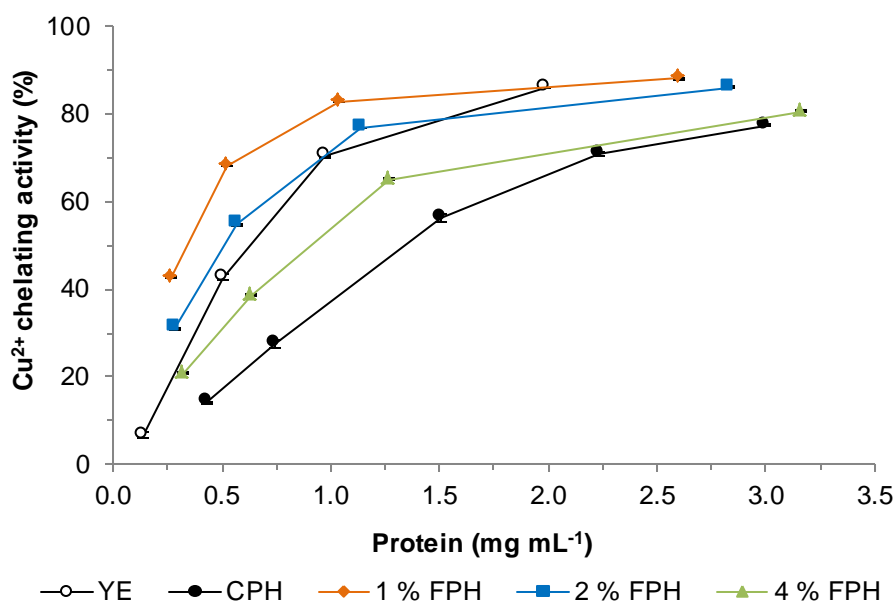


Figure 23. Cu^{2+} chelating activity of 1 %, 2 % and 4 % FPH at the initial time (0 hours), CPH and YE as a function of protein concentration.

The YE exhibited higher chelating activity than commercial CPH (fig. 23). The differences of chelating activity among FPH samples at time zero can be explained by the YE/CPH proportion. In fact, an increasing chelating activity was obtained with this increase of the YE/CPH proportion. This effect of the YE presence is particularly evident in 1 % FPH. A similar effect was also obtained for the DPPH radical scavenging activity.

In figure 24 is shown chelating activity of 1 % FPH at different fermentation times as a function of protein concentration. At the initial time 2 % FPH exhibited the best binding

capacity, when compared to the other fermentation periods. On the other hand, it seems that there are no major differences in the binding capacity of hydrolysates prepared after 24, 50 and 72 hours.

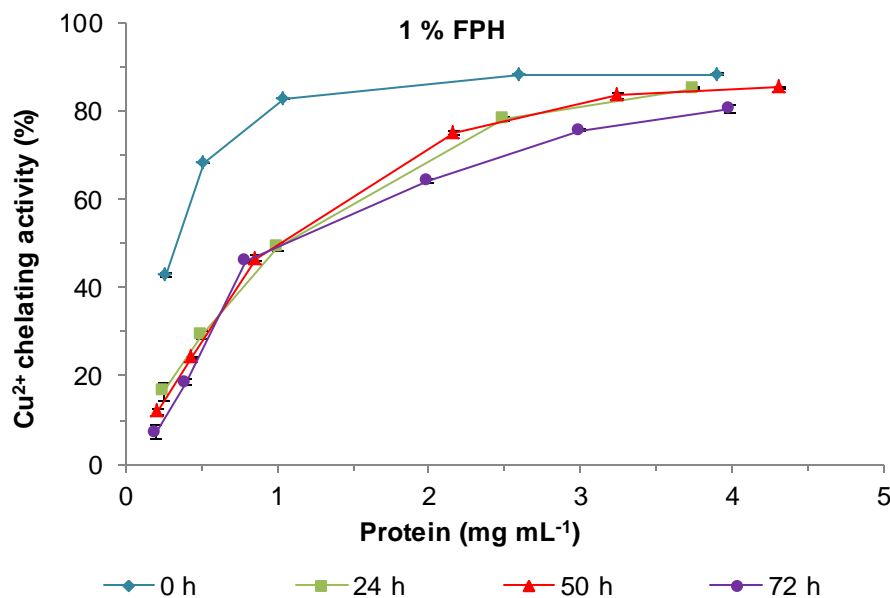


Figure 24. Cu²⁺ chelating activity of 1 % FPH samples at different fermentation periods

Similar to what happened in 1 % FPH, at the initial time 2 % FPH exhibited the best binding capacity, when compared to the other samples with 24, 50 and 72 hours of fermentation. However, the difference between time zero and the other fermentation periods do not seem as abrupt as in 1 % FPH (fig. 25).

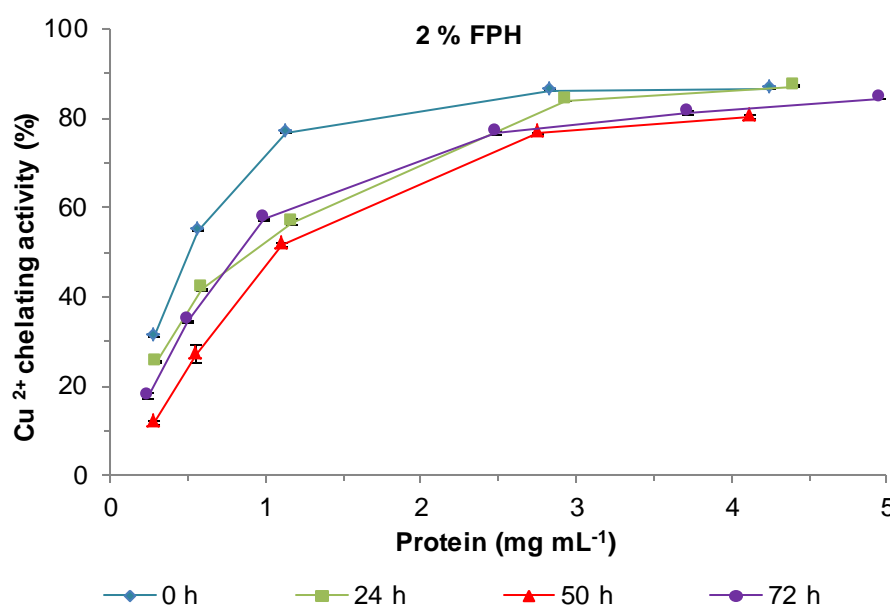


Figure 25. Cu²⁺ chelating activity of 2 % FPH samples at different fermentation periods.

As for 4 % FPH (fig. 26), the binding capacity at the different fermentation times seems to follow the exact same trend as in 2 % FPH.

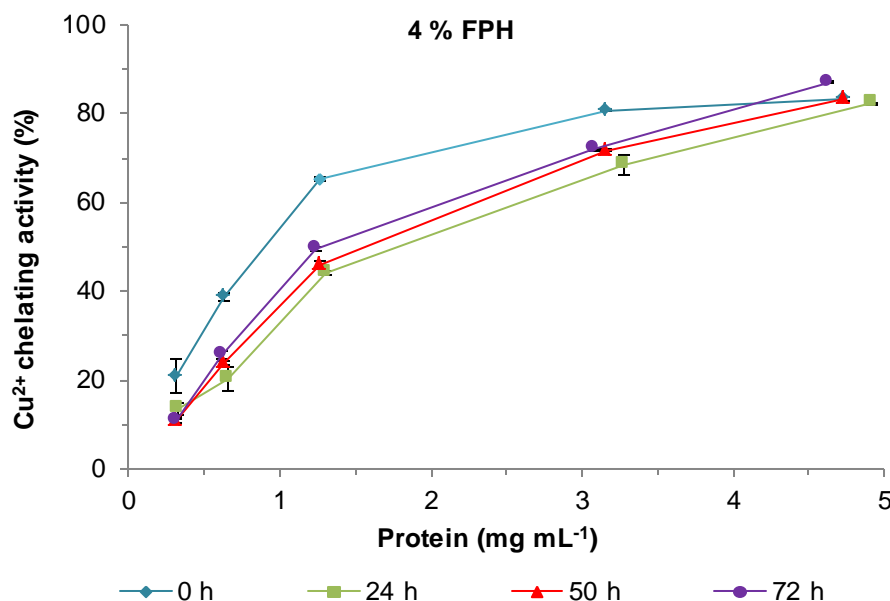


Figure 26. Cu²⁺ chelating activity of 4 % FPH samples at different fermentation periods.

In order to compare the Cu²⁺ chelating activity of all samples, a concentration of 1 mg FPH mL⁻¹ was chosen and represented as function of the fermentation period (fig. 27).

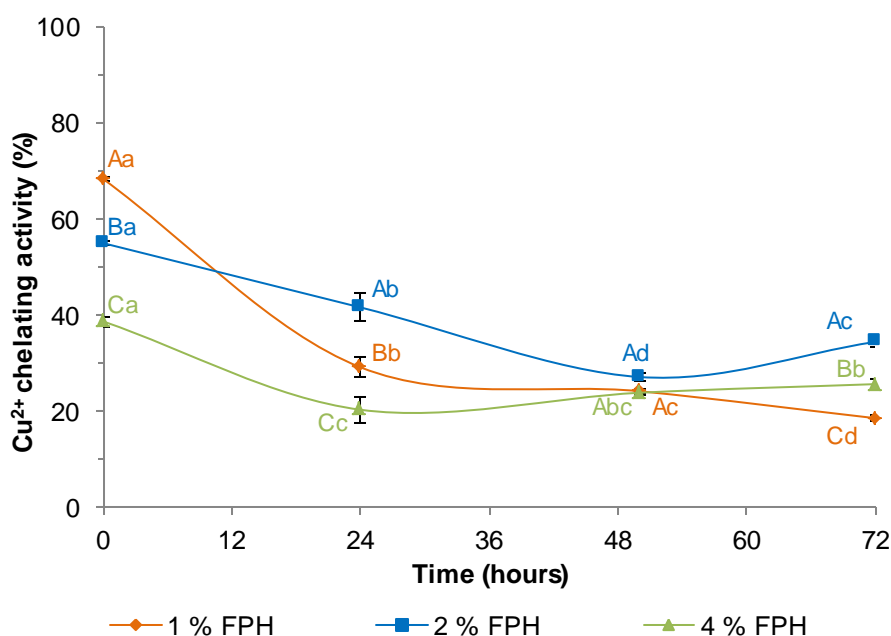


Figure 27. Evolution of Cu²⁺ chelating activity for all samples in 1 mg FPH mL⁻¹. The same letter indicates values not significantly different ($p > 0.05$), with A, B, C comparing the same fermentation time and a, b, c, d comparing the same FPH sample.

It can be seen that chelating activity decreased significantly ($p \leq 0.05$) from initial time to 72 hours of fermentation in all FPH. The highest decrease of Cu^{2+} binding capacity was recorded in 1 % FPH. This decrease is particularly evident between the initial value and after 24 hours of fermentation, which may result from the disappearance of the components of YE. On the other hand, the lower proportion of YE in 2 % and 4 % FPH led to a less accentuated decrease of chelating activity, when compared to 1 % FPH. The Cu^{2+} chelating activity values at 72 hours of fermentation were all significantly different ($p \leq 0.05$) and 2 % FPH was the one that presented better binding capacity.

According to Saiga *et al.* (2003) and Ovissipour *et al.* (2013), the decrease of Cu^{2+} binding capacity of protein hydrolysates may be justified by a low content in effective sites capable of chelating this metal ion, caused by the differences in the structure and length of the peptides. It was also reported by Zhu *et al.* (2008) that thiol and carboxylic groups are considered high-affinity metal binding sites, through electrostatic and ionic interactions with copper. Torres-Fuentes *et al.* (2011) stressed the importance of histidine in the chelation of Cu^{2+} and Carrasco-Castilla *et al.* (2012) related the decrease of Cu^{2+} binding capacity with the content of negatively charged amino acids, like aspartate and glutamic acid. The decrease of Cu^{2+} chelating activity of FPH could be related to changes of the amino acid profile of the peptides such as the loss of histidine.

4.5. Iron chelating activity

Like Cu^{2+} , Fe^{2+} is also involved in the production of active radicals resulting from the decomposition of hydrogen peroxide. Thus the effect of fermentation on the Fe^{2+} chelating activity was tested and the results obtained with the different FPH are shown in figure 28. For comparison the results of the chelating activity of commercial CPH and YE are also presented in this figure.

YE and CPH showed similar values for all the tested protein concentrations. On the other hand, they presented lower chelating activity than all FPH at initial time. The highest binding capacity was recorded in 1 % FPH and decreased with the increasing percentage of CPH. The sum of the individual chelating effect of each component of the different FPH was lower than the actual measured values. These results suggest that a synergistic effect between CPH and YE may occur. This effect was dependent on the YE/CPH ratio and decreased with decreasing value of this ratio.

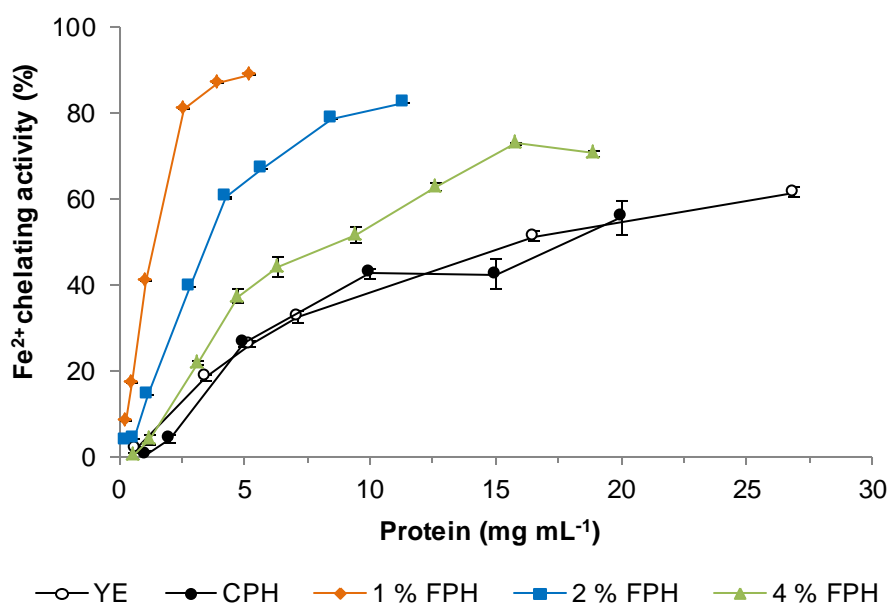


Figure 28. Fe²⁺ chelating activity of 1 %, 2 % and 4 % FPH, CPH and YE at the initial time (0 hours) as a function of protein concentration.

In figure 29 are presented the results of Fe²⁺ binding capacity for 1 % FPH, as function of protein concentration obtained after different fermentation periods.

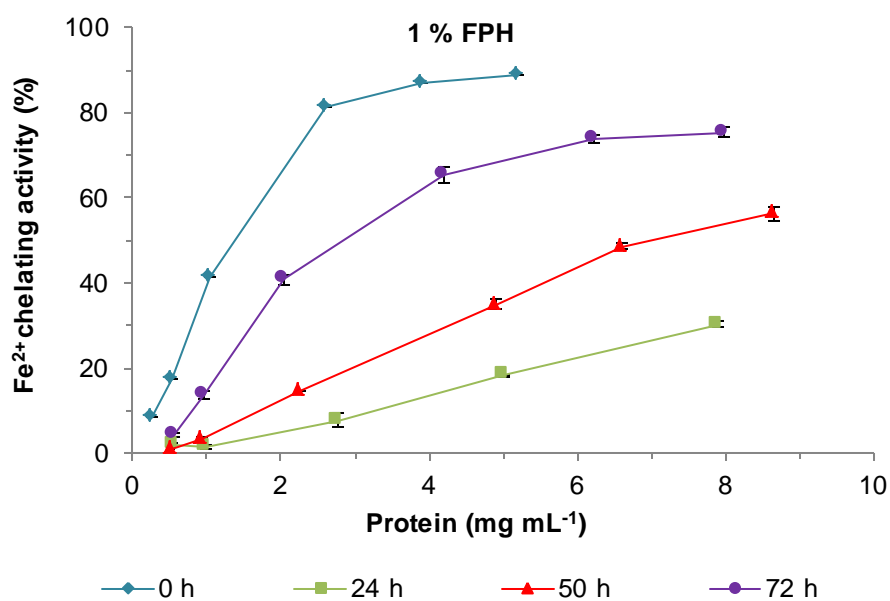


Figure 29. Fe²⁺ chelating activity of 1 % FPH samples at different times of fermentation.

As it can be seen, a considerable decrease of the chelating activity was recorded after 24 hours of fermentation. This result may be due to the consumption of YE constituents during this period and thus the synergistic effect between this constituents and CPH didn't occur in the FPH obtained after 24 hours. However, the FPH obtained after increasing fermentation periods (50 and 72 hours) presented an increasing binding capacity, which indicate that the new peptides formed had higher chelating activity.

The results of the 2 % FPH (fig. 30) also show a similar decrease of these property in the hydrolysates obtained after 24 hours. On the other hand, the increase of chelating activity of FPH obtained after 50 and 72 hours was not notorious as in the case of 1 % FPH. These results may be due to some dilution of the new active peptides in a matrix of intact peptides.

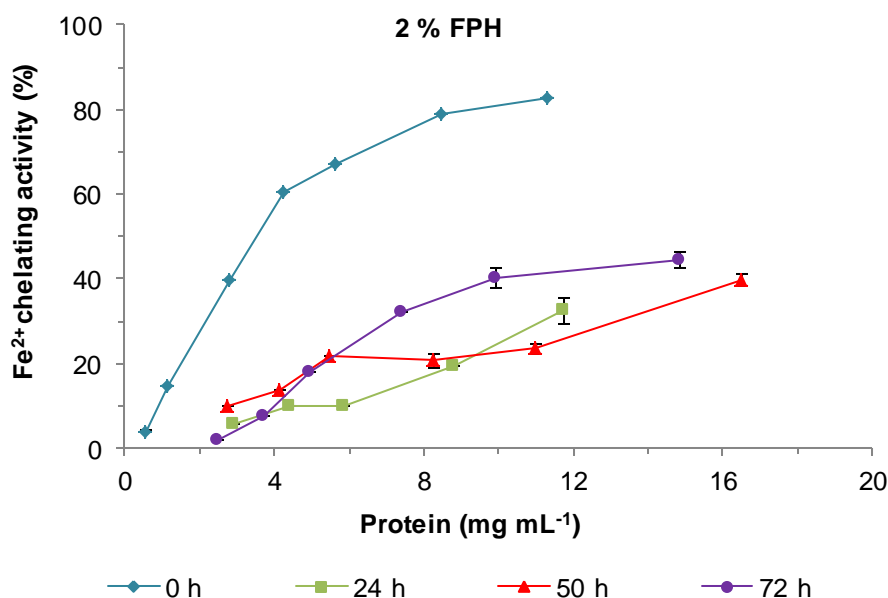


Figure 30. Fe²⁺ chelating activity of 2 % FPH samples at different times of fermentation.

In the case of 4 % FPH (fig. 31), the decrease of this capacity was not recorded after 24 hours, which may result from the lower synergistic effect previously mentioned. However, it was expected a relatively lower capacity than the 4 % FPH at the initial time.

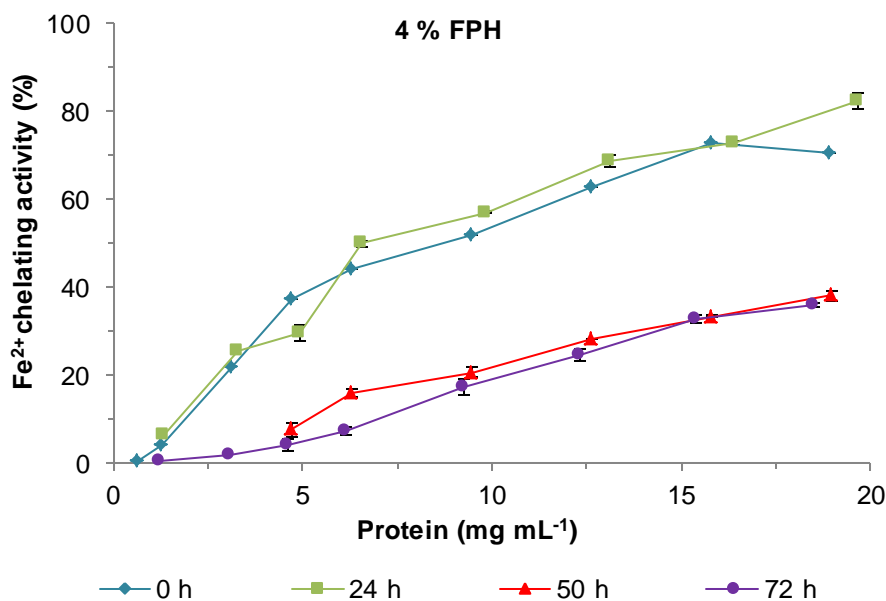


Figure 31. Fe²⁺ chelating activity of 2 % FPH samples at different times of fermentation.

In order to compare the Fe²⁺ chelating activity of the three FPH as a function of the fermentation period, a concentration of 10 mg FPH mL⁻¹ was chosen and the results are presented in Figure 32.

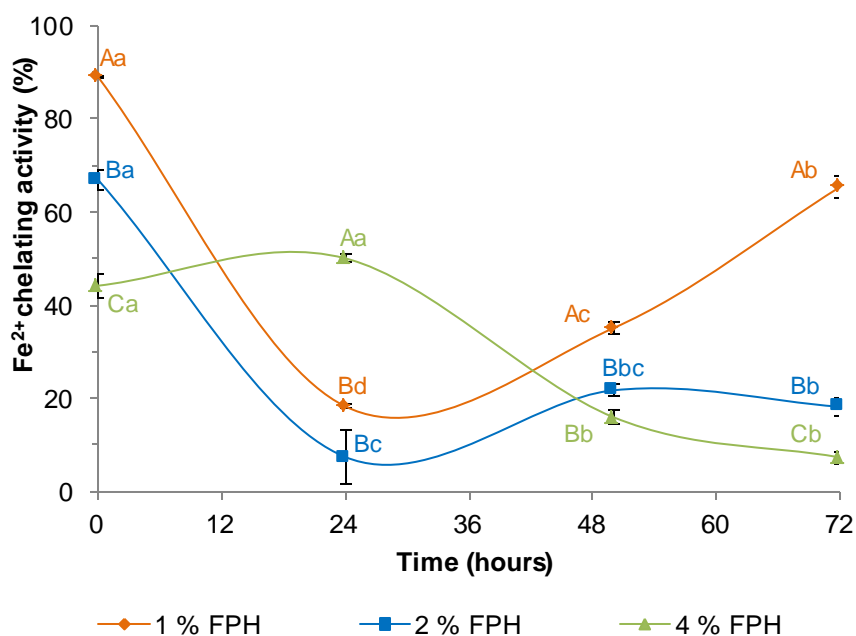


Figure 32. Evolution of Fe²⁺ chelating activity for all samples, when concentration is 10 mg FPH mL⁻¹. The same letter indicates values not significantly different ($p > 0.05$), with A, B, C comparing the same fermentation time and a, b, c, d comparing the same FPH sample

It's well evidenced the decrease of Fe^{2+} binding capacity in the 1 % and 2 % FPH prepared after 24 hours. It is also noticeable the increase of this capacity in the 1 % FPH after 50 and 72 hours. This increase was not observed in the 2 % FPH, as a result of the dilution of the active peptides formed. This dilution effect was even more evident in 4 % FPH, where the lowest chelating activity was measured.

The results obtained for the Fe^{2+} chelation by FPH didn't show a clear trend between this capacity and the fermentation periods, whereas in the case of Cu^{2+} chelation a general decrease of binding capacity with the fermentation time was observed. It's also to highlight the higher chelating activity of FPH for Cu^{2+} than for Fe^{2+} . Kong & Xiong (2006) also obtained lower Fe^{2+} chelating activity when compared to Cu^{2+} , which was related to a greater requirement of coordination sites to bind Fe^{2+} .

CHAPTER IV – CONCLUSION**1. CONCLUDING REMARKS**

This study revealed that it is possible to produce fermentative protein hydrolysates, using a marine proteolytic strain and fish protein hydrolysate. These new hydrolysates had a different peptide profile, enriched with smaller peptides. At the end of fermentation period (72 hours) all FPH presented peptides with MW higher than 400 Da (13 – 19 %), between 75 and 200 Da (24 – 30 %) and lower than 75 Da (51 – 63 %). The 4 % FPH was the hydrolysate with fewer changes, particularly lower percentage of smaller peptides.

Higher production of extracellular proteases was obtained in the culture medium with 2 % CPH, which may be due to a more balanced C/N ratio for this enzymatic production.

Based on the protein content, the highest yield (57.6 %) was achieved in the 4 % FPH.

The highest DH (26.76 %) was achieved in 2 % FPH, whereas in 4 % FPH only a small increase of DH (2.1 %) was obtained.

Concerning the antioxidant properties of FPH, it was concluded:

- The radical scavenging activity, evaluated by the DPPH test, was higher in 1 % and 2 % FPH prepared after 72 hours of fermentation. This may indicate a higher contribution of lipophilic peptides present in these hydrolysates;
- A significantly higher ABTS radical scavenging activity was recorded in 4 % FPH. Conversely, a significant decrease of this activity was observed in the other FPH;
- A synergistic effect between CPH and the YE constituents was recorded in the measurement of the reducing power of the FPH at the initial time. A linear increase of reducing power with the protein concentration was observed. Only this activity increased in all FPH;
- The copper chelating activity of FPH was significantly lower ($p > 0.05$) than the starting material. The increasing percentage of lower molecular weight peptides seems to negatively affect this property;
- The iron chelating activity of FPH was also significantly lower ($p > 0.05$) than the starting material. In this case, the increased percentage of smaller peptides had a positive effect on this property. These hydrolysates had an iron chelating activity considerably lower than the copper chelating activity.

In table 4 are summarized the results of antioxidant and chelating activities of all FPH.

Table 4. Summary of initial and final values of antioxidant activity obtained in all FPH.

Methodology	Sample / Measurement units	Fermentation period		Variation (%)
		0 hours	72 hours	
EC ₅₀ (mg protein mL ⁻¹)				
DPPH radical scavenging activity	1 % FPH	0.67 ± 0.03 ^{Bb}	0.30 ± 0.01 ^{Bc}	- 55.2
	2 % FPH	1.28 ± 0.03 ^{Aa}	0.40 ± 0.01 ^{Bc}	- 68.7
	4 % FPH	1.28 ± 0.01 ^{Ab}	1.13 ± 0.01 ^{Ab}	- 11.7
TEAC (mM)				
ABTS radical scavenging activity (5 mg FPH mL ⁻¹)	1 % FPH	2.60 ± 0.06 ^{Aa}	1.95 ± 0.02 ^{Bbc}	- 25.0
	2 % FPH	2.50 ± 0.07 ^{Aa}	2.22 ± 0.11 ^{Ab}	- 11.2
	4 % FPH	1.85 ± 0.08 ^{Bbc}	2.08 ± 0.02 ^{ABa}	+ 12.4
Ascorbic acid equivalent (µg mL ⁻¹)				
Reducing Power (10 mg FPH mL ⁻¹)	1 % FPH	83.18 ± 3.97 ^{Ab}	104.21 ± 0.76 ^{Aa}	+ 25.2
	2 % FPH	65.16 ± 0.34 ^{Bb}	96.11 ± 1.98 ^{Ba}	+ 47.4
	4 % FPH	49.87 ± 0.77 ^{Cb}	59.03 ± 0.86 ^{Ca}	+ 18.3
Chelating activity (%)				
Copper Chelating Activity (1 mg FPH mL ⁻¹)	1 % FPH	68.23 ± 0.61 ^{Ab}	18.55 ± 0.77 ^{Cd}	- 72.8
	2 % FPH	54.91 ± 0.36 ^{Ba}	34.40 ± 1.01 ^{Ac}	- 37.3
	4 % FPH	38.65 ± 1.21 ^{Ca}	25.63 ± 1.02 ^{Bb}	- 33.6
Chelating activity (%)				
Iron Chelating Activity (10 mg FPH mL ⁻¹)	1 % FPH	89.03 ± 0.12 ^{Aa}	63.06 ± 1.26 ^{Ab}	- 29.1
	2 % FPH	66.94 ± 2.20 ^{Ba}	18.16 ± 1.90 ^{Bb}	- 78.2
	4 % FPH	44.16 ± 2.70 ^{Ca}	7.29 ± 1.19 ^{Cb}	- 83.4

In general, 4 % FPH presented the lowest antioxidant properties, as a result of the dilution of the new formed peptides in an unchanged peptide matrix.

Taking into consideration the antioxidant properties and the concentration of hydrolysate at the end of the fermentation period it seems advisable using a starting material with the following proportions: 2 CPH : 0.5 YE : 0.5 sucrose and initial concentration of 3 % of these starting material.

2. FUTURE WORK

The identification of the strain used in this work would be important for a better understanding of the optimal growth conditions of this bacterium.

The enrichment of the culture medium with calcium salts could be advisable, having in mind that results on the characterization of the extracellular produced by this bacteria showed that it was activated by calcium ions.

In order to get a better understanding of the results of antioxidant activity, particularly the copper chelating activity, it is recommended to determine the amino acid composition of these hydrolysates. However, it would be necessary to know the peptide's structure to have a deeper knowledge of their antioxidant capacity.

The utilization of immobilized cells would be a step forward in order to take advantage of the proteolytic capacity of the strain.

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APPENDIXES

Appendix I. Standard curves

- *MW calibration curve for Superdex™ peptide 10/300 GL*

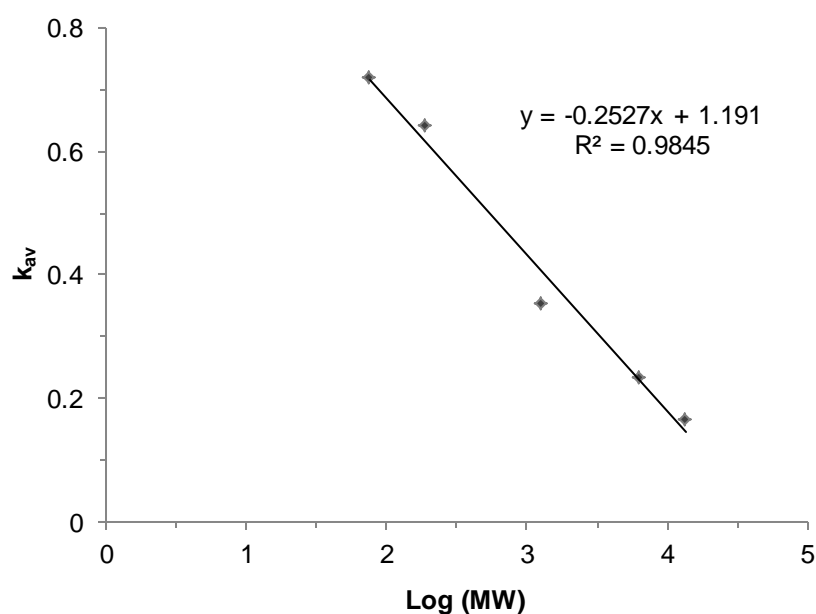


Figure 33. Calibration curve for Superdex™ peptide 10/300 GL, and its respective equation ($y = -0.2527x + 1.1909$, $R^2=0.989$, where y represents the k_{av} coefficient and x represents the logarithmic value of MW).

▪ **Trolox standard curve**

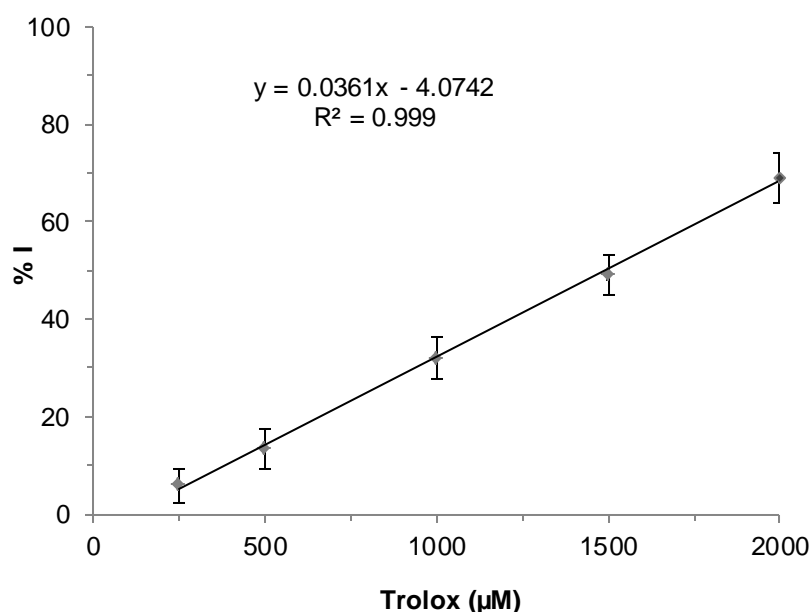


Figure 34. Trolox calibration curve and its respective equation ($y = 0.0361x - 4.0742$, $R^2=0.999$, where y represents the percentage of inhibition, or disappearance of ABTS, and x represents the concentration of Trolox).

▪ **Ascorbic acid standard curve**

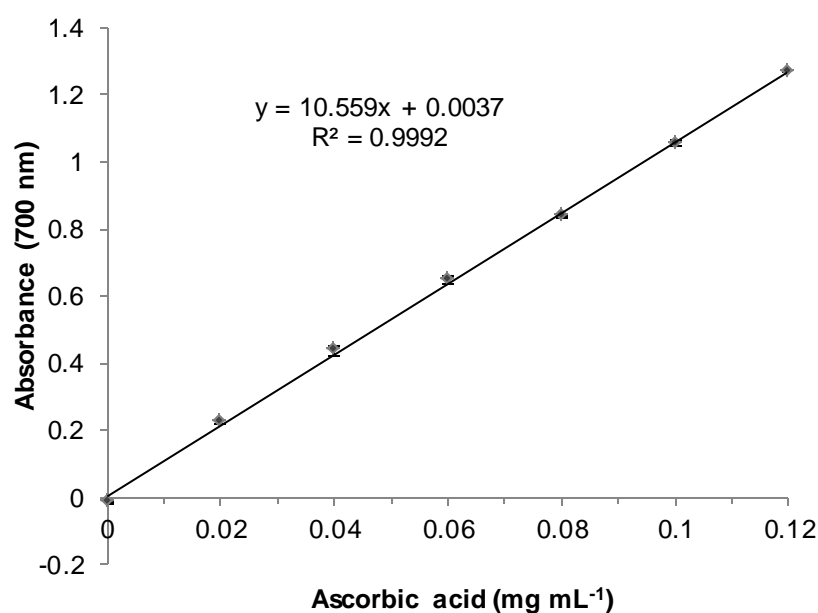


Figure 35. Ascorbic acid standard curve and its respective equation ($y = 10.559x + 0.0037$, $R^2= 0.9992$, where y represents the absorbance and x the concentration of ascorbic acid).

Appendix II. Molecular weights' index

▪ 1 % FPH

Table 5. List of MW of the peptides in 1 % FPH samples.

Fermentation period (hours)	Elution volume (mL)	k_{av}	Log (MW)	MW (Da)
0	14.5	0.41	3.11	1277.30
	17.98	0.62	2.25	175.89
	18.83	0.68	2.03	108.38
	20.29	0.77	1.67	47.17
	20.83	0.80	1.54	34.68
	21.32	0.83	1.42	26.23
	23.08	0.94	0.98	9.62
24	14.98	0.44	2.99	971.69
	18.81	0.68	2.04	109.62
	21.04	0.82	1.49	30.77
	21.82	0.86	1.30	19.73
	23.05	0.94	0.99	9.79
50	15.44	0.47	2.87	747.68
	18.85	0.68	2.03	107.15
	21.09	0.82	1.48	29.91
	21.81	0.86	1.30	19.84
	22.31	0.89	1.17	14.92
	23.78	0.99	0.81	6.46
72	15.58	0.47	2.84	690.36
	18.87	0.68	2.03	105.94
	21.05	0.82	1.49	30.59
	22.22	0.89	1.20	15.71
	24.13	1.01	0.72	5.29

▪ **2 % FPH**

Table 6. List of MW of the peptides in 2 % FPH samples.

Fermentation period (hours)	Elution volume (mL)	k_{av}	Log (MW)	MW (Da)
0	14.66	0.42	3.07	1166.02
	17.76	0.61	2.30	199.38
	18.79	0.67	2.04	110.88
	20.39	0.77	1.65	44.56
	21.01	0.81	1.50	31.30
	21.70	0.86	1.32	21.13
	22.99	0.94	1.01	10.13
24	15.13	0.45	2.95	892.10
	18.76	0.67	2.05	112.79
	21.13	0.82	1.47	29.23
	22.65	0.92	1.09	12.30
50	15.37	0.46	2.89	778.10
	18.85	0.68	2.03	107.15
	21.18	0.82	1.45	28.41
	22.78	0.92	1.06	11.42
72	15.56	0.47	2.84	698.27
	18.87	0.68	2.03	105.94
	21.10	0.82	1.47	29.74
	23.43	0.96	0.90	7.88
	24.82	1.05	0.55	3.57

▪ 4 % FPH

Table 7. List of MW of the peptides in 2 % FPH samples.

Fermentation period (hours)	Elution volume (mL)	k_{av}	Log (MW)	MW (Da)
0	14.98	0.44	2.99	971.69
	17.57	0.60	2.35	222.17
	18.69	0.67	2.07	117.38
	21.16	0.82	1.46	28.74
	22.19	0.89	1.20	15.98
24	14.97	0.44	2.99	977.25
	18.70	0.67	2.07	116.71
	21.13	0.82	1.47	29.23
	22.86	0.93	1.04	10.91
50	15.28	0.46	2.91	819.03
	18.86	0.68	2.03	106.54
	21.17	0.82	1.46	28.57
	23.53	0.97	0.87	7.45
72	15.35	0.46	2.90	787.01
	18.83	0.68	2.03	108.38
	21.27	0.83	1.43	26.99
	23.88	0.99	0.79	6.10

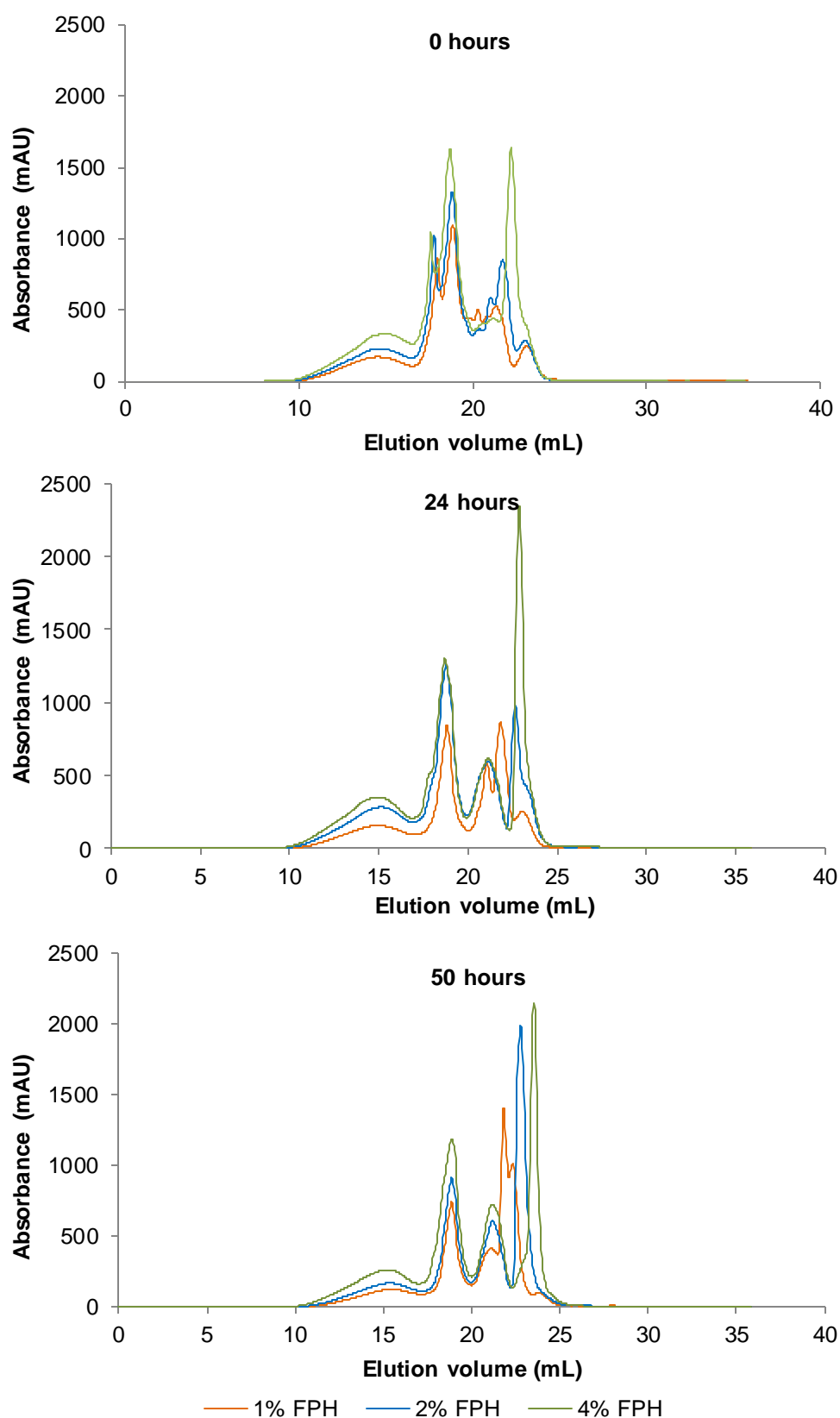
Appendix III. FPLC chromatograms

Figure 36. Gel filtration chromatograms showing the molecular weight distribution of hydrolysates at 0, 24 and 50 hours of fermentation.

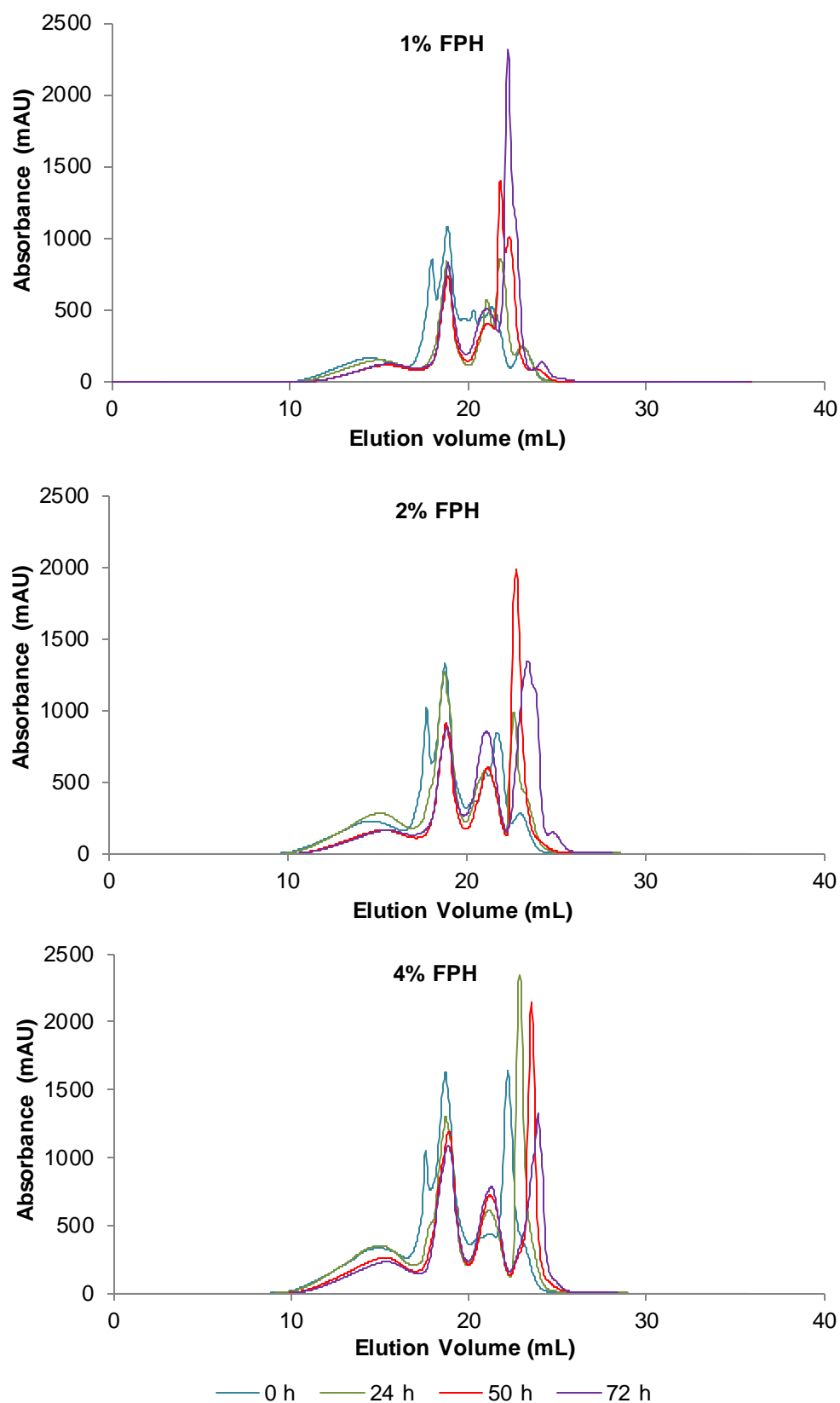


Figure 37. Gel filtration chromatograms showing the evolution of molecular weight distribution of 1 %, 2 % and 4 % FPH.

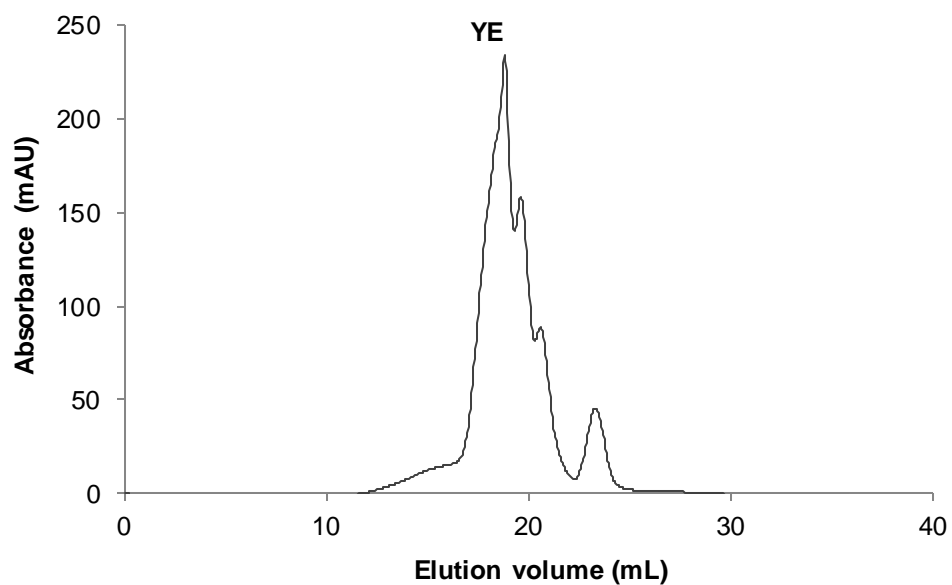


Figure 38. Gel filtration chromatogram showing the molecular weight distribution of YE.

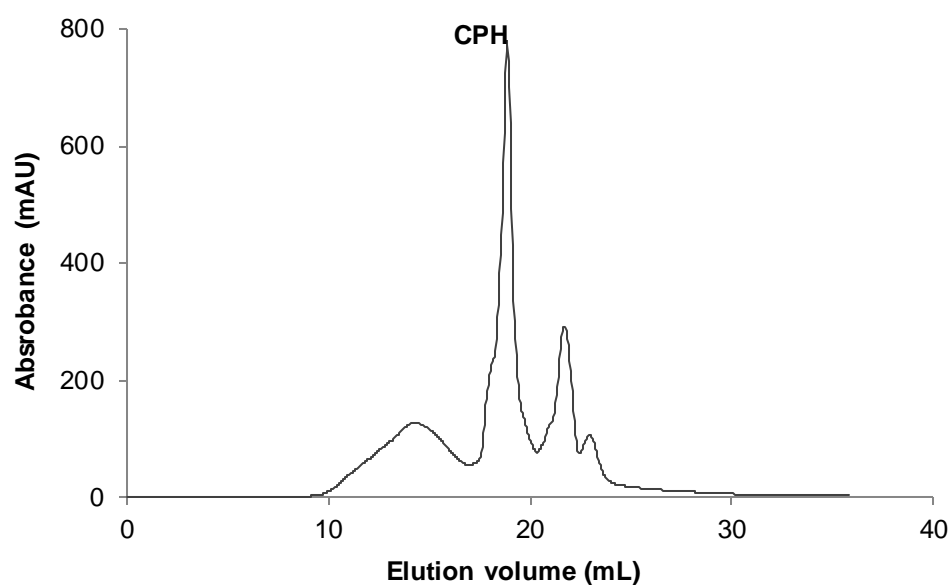


Figure 39. Gel filtration chromatogram showing the molecular weight distribution of CPH.